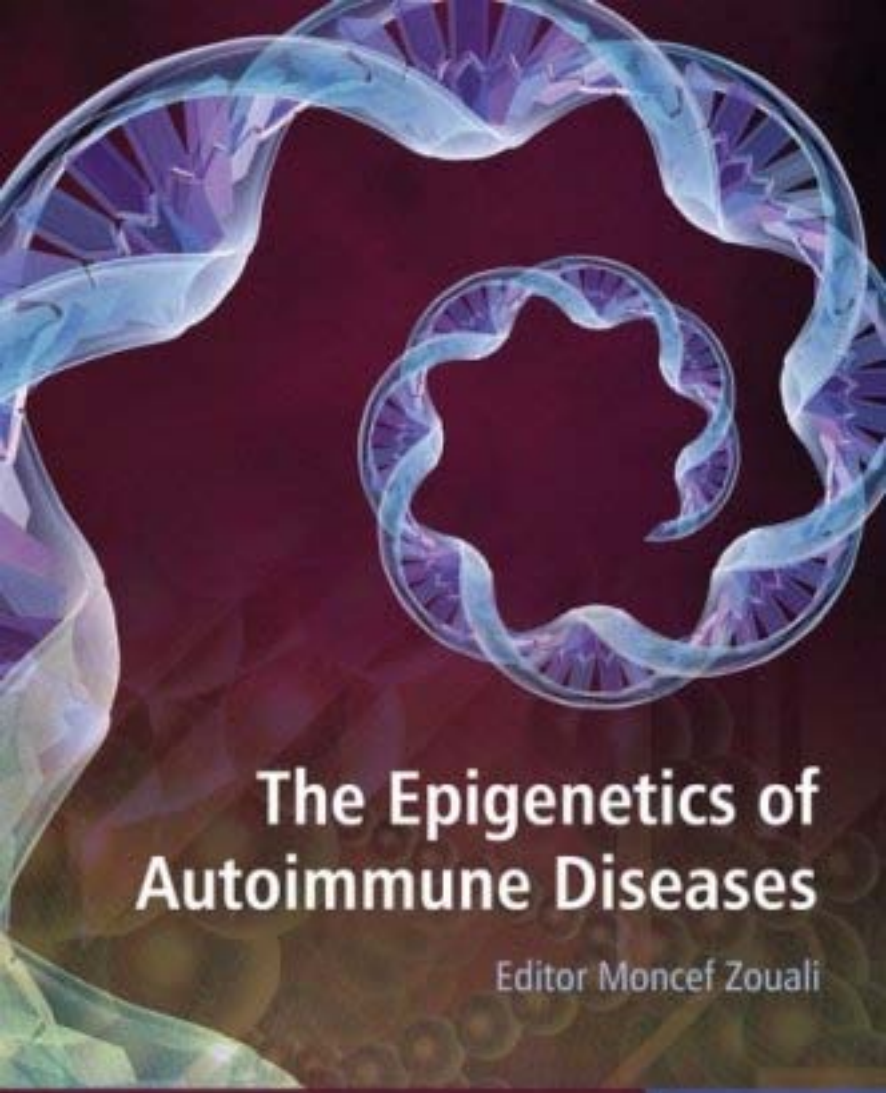

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Editor Moncef Zouali

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The Epigenetics of Autoimmune Diseases

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Editor

Moncef Zouali

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Preface

Investigation of the genome of several metazoan species revealed that the DNA sequences in all cells of a given individual are essentially the same, implying that genetic information, by itself, cannot fully account for the differential gene expression during cell differentiation and development. Parallel studies in the past decade also have unveiled the importance of an array of intricate epigenetic mechanisms that regulate transcription by affecting chromatin conformation –including DNA cytosine methylation, covalent modifications of histones, and certain aspects of RNA interference. Histones, for example, comprise a large number of residues that can be modified through several mechanisms, *i.e.* methylation, acetylation, phosphorylation, and ubiquitination. Such multiple, combinatorial modifications can give rise to highly complex patterns that can directly alter chromatin structure. Other modifications potentially serve as binding platforms to recruit additional effectors. The description of such a diversity of epigenetic factors led to several definitions of epigenetics. Recently, Adrian Bird proposed a revised definition that embodies contemporary usage of epigenetics: "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states".

Epigenomic studies performed largely as a result of the availability of powerful high-throughput tools, *i.e.*, high-density whole-genome microarrays, indicate that from the morphology of flowers to the eye color of insects, a variety of biological properties can be shaped by epigenetic influences. Investigation in different species also revealed that eukaryotic organisms can respond to environmental exposure by producing different phenotypes from the same DNA genome, implying that environmental stimuli could alter the state of the epigenome and affect gene expression by modifying DNA methylation and histone acetylation patterns. A striking example of this phenotypic plasticity has been reported in social insects by Ryszard Maleszka and co-workers. Thus, *Apis mellifera*, or honeybees, differentially feed genetically identical female larvae to create mainly workers and, when required, a limited number of queens. Despite their clonal identity at the DNA level, workers and queens differ markedly in morphological and physiological features, and exhibit contrasting reproductive capabilities, diverse life spans, and different behavioral repertoires. Remarkably, the honeybee has a full complement of all three functional DNA cytosine-5-methyltransferases with *in vivo* properties similar to those of the CpG methylation system in vertebrates. Its genome also encodes conserved methyl-binding proteins that include components of the nucleosome remodeling and histone deacetylase complex. This elaborate epigenetic

network that maintains the production of distinct adult morphologies, varied reproductive and behavioral systems, social organization and division of labor was demonstrated to be controlled by DNA methylation, a key epigenetic mark.

In rodents, such as the agouti mouse, a number of contrasting phenotypes, such as yellow and obese or brown and slim, can be controlled by varying the mother′s diet before, during, and after pregnancy. Not only can gouti gene expression be silenced by DNA methylation, but its magnitude is variable in genetically identical individuals because of epigenetic modifications established during early development. In the rat, it appears that maternal reproductive tactics can alter the function of the neuroendocrine system associated with female sexual behavior and maternal behavior. Such maternal effects are mediated by epigenetic modifications at the promoter of estrogen receptor alpha and subsequent effects on gene expression, potentially underlying the coordinated variation in multiple forms of reproductive function and distinct reproductive strategies. Differential maternal behavior in the female rat also was found to alter the methylation status of the promoter of the glucocorticoid receptor of her pups. Even in psychopathology, there is evidence that interactions between genes and the environment can influence behavior. For example, experimental studies have shown that early life rearing experiences in rodents can alter gene expression, and that this epigenetic "reprogramming" involves specific genes as well as specific environments linked to later behavior.

In humans, it is remarkable that monozygotic twins do not always show the same disease susceptibility, suggesting that epigenetic differences can give rise to disease predisposition. The recognition of epigenetics as an important factor in health and disease has led to a surge in research aimed at uncovering epigenetic factors underlying pathogenesis. Epidemiological observations revealed that, in children, cardiovascular and diabetes mortality can be influenced by the nutritional status of their parents and grandparents. It has even been proposed that psychotropic drugs can rewrite the epigenetic code of the brain by switching on, or off, a number of genes through epigenetic modifications. The large amount of "epigenomic" data generated also is providing new insight into the mechanisms and functions of several regulatory pathways, and is broadening our understanding of aging and a variety of disorders, such as cancer development.

Until recently, epigenetic regulation of the immune system was considered of little importance. However, recent studies demonstrated that highly developed epigenetic mechanisms take part in several aspects of the development of immune cells and in the generation of innate and adaptive immune responses. Importantly, epigenetic changes represent suitable targets for the prevention and treatment of disease, implying that, potentially, they can be reversed by potent drugs. A detailed understanding of such mechanisms therefore can lead to novel therapeutic strategies based on manipulation of this previously unexploited facet of immune regulation.

Human autoimmune diseases represent a group of complex disorders that affect 5–10 % of the world population. They can target virtually any organ and become life threatening. Their origin remains under scrutiny and the available treatments lack specificity and are associated with undesirable effects. Drawing on the research of

leading experts, *The Epigenetics of Autoimmune Disease* provides insights into a new area of autoimmunity. Firstly, it shows how highly developed epigenetic mechanisms take part in several aspects of normal immune regulation and in maintaining immune tolerance to self-determinants. Secondly, a number of chapters delve into specific epigenetic aspects of human autoimmune diseases –including multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, systemic sclerosis, autoimmune diabetes, thyroid autoimmunity, inflammatory bowel disease, and autoimmune hepatitis. In some of them, it is striking that the environment can prompt epigenetic changes that might influence autoimmunity development. Finally, this volume illustrates how understanding epigenetic mechanisms can lead to therapeutic strategies based on manipulation of this novel facet of immune regulation. Because epigenetic markers are easier to modify than the underlying sequences, they can be targeted for treatment. Discussed in this volume are therefore novel approaches that are being investigated to prevent or treat autoimmune diseases.

By providing a comprehensive review of epigenetic regulation of immune tolerance to self-antigens and its deregulation, I hope that this work will reveal new directions for future research in autoimmunity research. I also would like to thank the contributors to this volume for their patience and collegiality.

Moncef Zouali
Paris, September 2008

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PART I

Transcription Factors: Partners of Immune Tolerance to Self

The Epigenetics of Autoimmune Diseases Edited by Moncef Zouali
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1

Transcriptional regulation of T cell tolerance

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1.1 Introduction

The immune system has a difficult task, it must recognize a diverse array of pathogens to protect the individual, and at the same time limit its recognition of self-tissues. Lymphocytes are capable of these functions by somatically rearranging their antigen receptor genes. This process of generating antigen-specific lymphocytes has its limitations. It can produce self-reactive cells, which, if left unchecked, can lead to the development of autoimmune diseases. Controlling this process occurs at two different levels: central tolerance and peripheral tolerance. Both self-reactive B and T lymphocytes can be eliminated or rendered tolerant, but this chapter will focus only on T cells.

T cell precursors migrate from the bone marrow to the thymus where they develop and are selected based on antigen recognition. Putative self-reactive thymocytes bearing antigen receptors that bind strongly to self-antigens presented by thymic epithelial and dendritic cells are negatively selected and induced to undergo apoptosis [1]. Nevertheless, negative selection of self-reactive thymocytes is not completely effective and mature T cells that carry self-reactive T cell receptors (TCRs) may escape to the periphery. There, both cell intrinsic and extrinsic mechanisms exist to limit T cell activation against self-antigens [2–5]. We will discuss two major mechanisms that maintain peripheral T cell tolerance: anergy (intrinsic) and regulatory T cells (extrinsic); and analyse how different levels of transcriptional regulation underlie the mechanisms responsible for the inactivation or suppression of self-reactive T cells.

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1.2 T cell anergy

T cell anergy refers to a cell-intrinsic mechanism that renders a T cell functionally inactive. The term clonal T cell anergy was introduced by Schwartz and colleagues in 1987, who observed that previous stimulation with chemically fixed antigen-presenting cells generated hypo-responsive $CD4^+$ T cell clones that failed to produce interleukin (IL)-2 and proliferate upon re-stimulation [6]. It was later found that this treatment prevented B7 from interacting with CD28, which suggested that proper T cell activation required signalling provided by co-stimulation in addition to TCR and peptide-bound major histocompatibility complex (MHC) signalling. These results were supported by other studies showing that the absence of the co-stimulatory signals induced a state of anergy [7–9] and led to the model that T cells are rendered anergic by activation of the TCR alone (signal 1) in the absence of co-stimulation (signal 2). Anergy can also be established *in vitro* by occupancy of the TCR by partial agonist peptides or by stimulating T cells with low concentrations of agonist peptides even in the presence of co-stimulation [10,11]. In all models, clonal anergy is induced as a consequence of partial or suboptimal activation of T cells.

Several *in vivo* models of anergy have also been described, including systemic administration of T cell superantigens and oral tolerance, which induces T cell anergy when antigen is administered at high doses. In addition, tolerance can also be induced *in vivo* by the injection of soluble antigen into mice that had been adoptively injected with TCR-transgenic T cells specific for that antigen, by the transfer of T cells bearing TCRs that recognize a given antigen into mice with endogenous expression of this antigen, or in double transgenic mouse models that express a unique TCR and its cognate antigen [12]. The common feature of the various models of T cell tolerance, including the *in vivo* models that defined what has been termed adaptive tolerance, is that they lead to the induction of a hypo-responsive state in T cells, characterized by defective IL-2 production and hypo-proliferation upon activation. Pre-activation of T cells seems to always be necessary before the hypo-responsive state can be established. However, detailed analysis of these tolerance-inducing conditions has revealed intrinsic differences that may represent specific states of T cell unresponsiveness [12]. For instance, clonal anergy (*in vitro* anergy) represents a general growth-arrest state in previously activated T cells accompanied by profound defects in the production of IL-2 and other cytokines. This state of unresponsiveness to antigen is long-lasting and can be rescued by addition of exogenous IL-2. On the other hand, adaptively tolerized T cells are also hypo-responsive and show an almost complete inability to secrete cytokines upon antigen re-encounter. However, to maintain this status, which cannot be reversed by IL-2 receptor signalling, persistent exposure to antigen is required [13]. Evidence also indicates that cytotoxic T lymphocyte antigen 4 (CTLA-4) signalling could play an important role in the induction of adaptive tolerance: blocking CTLA-4 prevents anergy induction in T cells [14] and $CTLA\text{-}4^{-/-}$ T cells are resistant to tolerance induction [15]. These results suggest a critical role of CTLA-4 in controlling T cell tolerance *in vivo*; however, CTLA-4 does not seem to be necessary for the induction of clonal T cell anergy. Recent evidence has also identified an important role for adenosine

in the control of peripheral tolerance. In response to an anergizing stimulus, T cells upregulate the expression of the adenosine receptor A_{2A}. Engagement of this receptor by free adenosine leads to the blockage of the Ras/mitogen-activated protein kinases (MAPKs) pathway and the establishment of anergy in T cells [16].

We will discuss recent evidence that elucidates the mechanisms of transcriptional regulation that control the induction and maintenance of T cell anergy. Although most of these studies have been carried out to explain the induction and maintenance of T cell clonal anergy, some of these findings have also been shown to apply to other models of *in vivo* T cell tolerance.

1.3 Ca^{2+}/calcineurin/NFAT signalling in T cell anergy

T cell activation is a dynamic process. The cell-adhesion molecules lymphocyte function-associated antigen-1 (LFA-1) and intercellular cell-adhesion molecule 1 (ICAM-1) form a stable bridge between the T cell and the antigen-presenting cell, which will display a peptide-bound MHC to the TCR (signal 1). In addition, proper T cell activation also requires engagement of co-stimulatory signals (signal 2). A number of co-stimulatory receptor–ligand pairs have been described; however, the CD28–B7.1/B7.2 pair is the best characterized [17]. Interactions through these receptors activate signalling pathways that polarize the T cell and develop an organized immunological synapse with a dense area of TCR–MHC and co-stimulatory receptor–ligand interactions [18]. Propagation of these signals leads to downstream intracellular events that allow proper T cell activation characterized by the ability to proliferate, produce cytokines and gain effector functions (Figure 1.1).

Stimulation of the TCR leads to a number of signalling events that activate the membrane-bound phospholipase Cγ (PLC-γ) that cleaves phosphatidylinositol 4,5-bisphosphate to inositol 3-phosphate and diacylglycerol (DAG), which signal independently through different pathways. Inositol 3-phosphate increases cytoplasmic levels of calcium by releasing intracellular stores from the endoplasmic reticulum. The elevated calcium levels trigger calcium-release-activated channels in the plasma membrane to open, which allows extracellular calcium to enter the cell, presumably to sustain high levels of calcium [19]. The calcium-dependent protein calmodulin binds calcium and activates the phosphatase calcineurin. Calcineurin in turn dephosphorylates members of the nuclear factor of activated T cells (NFAT) family of transcription factors, which translocate to the nucleus and cooperate with other transcription factors to promote the expression of an activation-induced transcriptional programme that includes many cytokine genes [20]. NFAT is a family of transcription factors ubiquitously expressed in numerous tissues [21,22]. NFAT family members include NFAT1 (NFATc2, NFATp), NFAT2 (NFATc1, NFATc), NFAT3 (NFATc4), NFAT4 (NFATc3, NFATx) and the distantly related NFAT5 (TonEBP) [20,23,24]. NFAT1–4 are regulated by calcineurin through intracellular calcium fluxes as described above; NFAT5 is regulated by osmotic stress. Upon dephosphorylation, a nuclear localization signal is exposed in the calcineurin-regulated

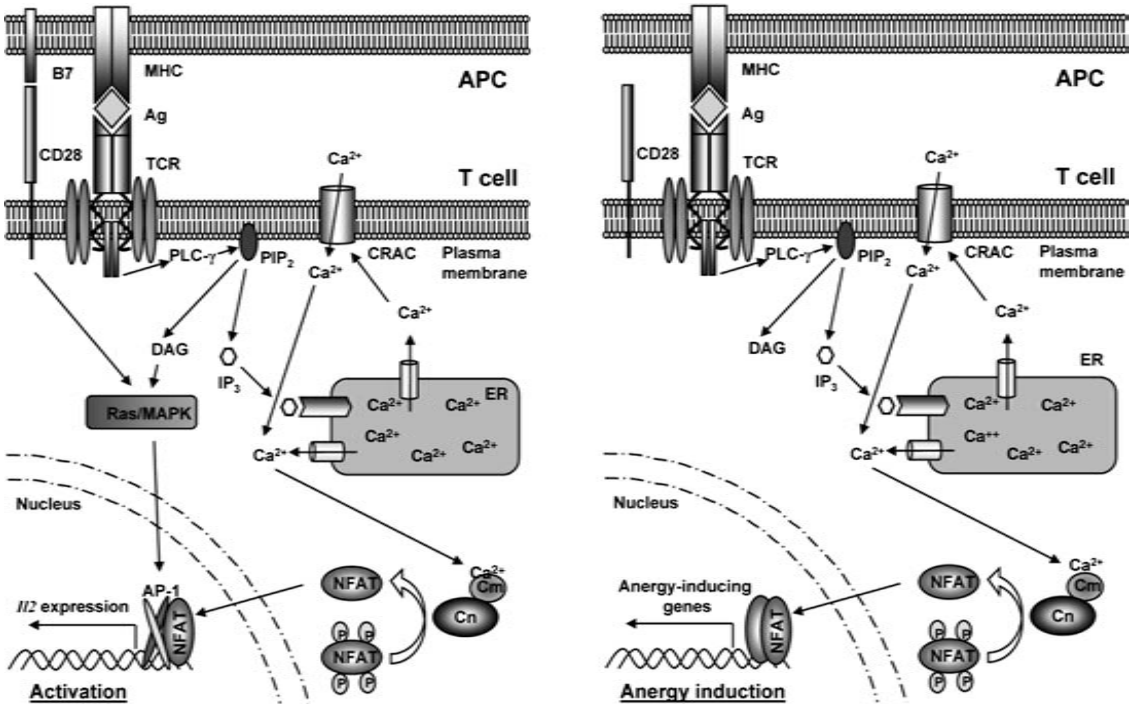

Figure 1.1 NFAT proteins regulate different programmes of gene expression in T cells: activation (left) versus anergy (right). Engagement of the TCR to a cognate peptide–MHC presented on the surface of antigen-presenting cells leads to the activation of receptor-associated tyrosine kinases that in turn leads to the activation of phospholipase-Cγ (PLC-γ) that cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 3-phosphate (IP_3) and diacylglycerol (DAG). IP_3 receptor activation in the endoplasmic reticulum (ER) leads to release of intracellular stores of calcium. This transient elevation in Ca^{2+} opens calcium-release-activated channels (CRACs) in the plasma membrane, allows an inward flux of Ca^{2+} from the extracellular compartment, and sustains high levels of intracellular calcium. Calmodulin (Cm) binds Ca^{2+} and activates the phosphatase calcineurin (Cn) to dephosphorylate NFAT. This dephosphorylation localizes NFAT to the nucleus. Engagement of co-stimulatory receptors, such as CD28, cooperates with signals initiated at the TCR (e.g. DAG-mediated recruitment of the Ras guanine-exchange factor RagGrp1) to activate the Ras/MAPK pathway that leads to the induction of the expression and nuclear translocation of Fos and Jun to form the activator protein 1 (AP-1) transcriptional complex. NFAT and AP-1 cooperatively activate transcription of many cytokine genes, including *Il2*. In the absence of co-stimulation, NFAT dimers, and possibly other NFAT-containing complexes, direct the expression of a specific set of genes. Proteins encoded by those genes inhibit T cell function at different levels and induce a status of T cell anergy.

NFAT proteins and translocates into the nucleus [20,23]. Nuclear NFAT interacts with other transcription factors, including the well-characterized interaction with activator protein 1 (AP-1) complexes (Fos and Jun heterodimers), to induce the expression of many cytokines in activated T cells [25,26]. Clonal anergy is induced when stimulation of the TCR occurs in the absence of co-stimulatory signals (Figure 1.1). In this scenario, NFAT proteins localize to the nucleus, but AP-1 complexes are not properly formed, as full activation of the Ras/MAPK pathways, which control the activation of Fos and Jun proteins, requires co-stimulation. In the absence of AP-1 cooperation, NFAT1 has been shown to induce the expression of specific genes encoding known or presumed negative regulators of T cell activation [27]. These anergy-associated genes are involved in a variety of mechanisms to maintain the anergic state, which include, among others, epigenetic modifications of the IL-2 promoter that lead to the silencing of the expression of this gene [28–30].

1.4 Transcriptional programme of T cell anergy

Early insights into clonal T cell anergy indicated the requirement for new protein synthesis to maintain the anergic state, as treatment with cycloheximide blocked the induction of anergy [7]. Genome-wide studies both *in vitro* [27] and *in vivo* [31] have demonstrated that T cell anergy is an active process that results from a distinct transcriptional profile that results in the expression of a set of anergy-inducing genes (Figure 1.2). As discussed above, one of the transcription factors responsible for the expression of many anergy-associated genes are NFAT proteins. Blocking calcineurin-mediated activation of NFAT proteins with cyclosporin A prevents the induction of T cell anergy, whereas T helper (Th) 1 cells derived from NFAT1$^{-/-}$ mice are less susceptible to anergizing stimuli and fail to upregulate the expression of many anergy-associated genes. Furthermore, expression of a constitutively active form of NFAT1 induces the expression of several anergy-associated genes, suggesting that, at least for those genes, complexes containing only NFAT proteins are responsible for their transcription [27,32,33]. Recent evidence from our laboratory indicates that, in response to anergizing stimuli, NFAT1 homodimers may form and induce the transcription of genes involved in maintaining a hypo-responsive state.

NFAT proteins are not the only transcription factors that regulate the expression of anergy-inducing genes. The members of the early growth response (Egr) family of transcription factors Egr2 and Egr3 are also associated with the induction of T cell anergy. Both genes have been shown to be preferentially expressed in anergic T cells and evidence indicates that their expression is required to downregulate TCR signalling in anergic cells. In fact, Egr3-deficient T cells are resistant to anergy induction *in vivo* and the ectopic expression of Egr2 or Egr3 results in inhibition of IL-2 expression [34]. Egr2 and Egr3 are probably targets of NFAT, as their expression is blocked by cyclosporin A. The observation that *Egr3*$^{-/-}$ T cells fail to upregulate the expression of Cbl-b (see below) in response to calcium signals suggests that Egr proteins are also involved in controlling the transcription of anergy-inducing genes [34]. Expression of

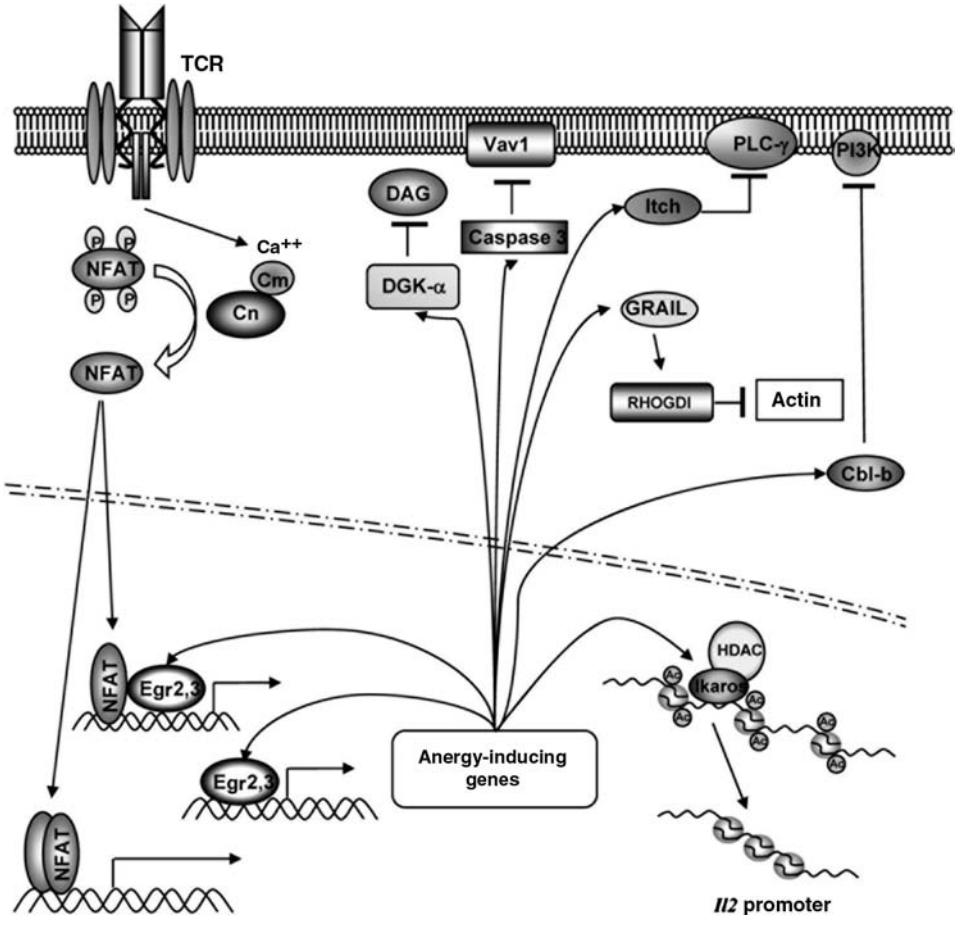

Figure 1.2 Epigenetic modifications contribute to the inhibition of the activation-induced IL-2 expression in anergic T cells. In response to anergizing stimuli, Ca^{2+}-/calmodulin (Cm)-/calcineurin (Cn)-mediated activation of NFAT proteins results in the induction of an anergy-associated programme of gene expression. Among these genes, the members of the early growth response (Egr) family of transcription factors Egr2 and Egr3 in turn contribute, in cooperation with NFAT or in an independent manner, to activate the expression of more anergy-inducing genes. The proteins encoded by those genes include several E3-ubiquitin ligases, such as Itch, Gene Related to Anergy In Lymphocytes (GRAIL) and Cbl-b, caspase 3 and diacylglycerol kinase-α (DGK-α), causing dampening of TCR signalling by interfering with TCR signalling at different levels. Itch has been shown to ubiquitinate PLC-γ1 and the protein kinase C θ targeting them for degradation. Cbl-b ubiquitinates the p85 subunit of the phosphoinositide-3-kinase (PI3K) preventing its association with CD28. GRAIL ubiquitinates and stabilizes RhoGDI which sequesters Rho family GTPases away from the membrane, preventing their effects on the reorganization of the cytoskeleton. Caspase 3 directly targets and inactivates Vav1, whereas DGK- α depletes available DAG by converting it into phosphatidic acid. The transcription factor Ikaros is also expressed in anergic T cells where it binds to the *Il2* promoter, recruiting histone histone deacetylases (HDACs) that remove acetyl (Ac) groups from histones 3 and 4 in this locus, leading to the establishment of epigenetic changes that result in stable silencing of the *Il2* gene expression.

Egr2 and Egr3 may therefore result from NFAT activation in response to anergizing stimuli. These transcription factors would then activate the expression of other anergy-inducing genes, such as Cbl-b. Whether Egr2 and Egr3 can induce the expression of Cbl-b as independent complexes or in cooperation with NFAT remains to be determined.

Anergic T cells upregulate, thus, the expression of genes that account for the block in the TCR signalling cascade through the activation of several mechanisms that include degradation or inactivation of key signalling molecules [35,36]. The gene related to anergy in lymphocytes (GRAIL) is a transmembrane RING finger protein that was identified in T cell clones stimulated with MHC–peptide in the absence of co-stimulation [37]. GRAIL mRNA and protein are highly upregulated in response to tolerizing stimuli in a calcium-/calcineurin-dependent manner. Functionally, GRAIL has been described to function as an E3 ubiquitin ligase *in vitro* [37] and, when over-expressed in T cells, GRAIL is sufficient to abrogate IL-2 production and inhibit proliferation *in vitro* [38]. Although a GRAIL-deficient mouse has not been reported yet, experiments performed in mice using bone marrow chimaeras with cells expressing an enzymatically inactive dominant negative form of GRAIL indicate that this E3 ligase is required for the generation of peripheral T cell tolerance *in vivo* [39]. Similarly, over-expression of Otubain1, which induces the degradation of GRAIL, promotes resistance to the induction of T cell anergy [38]. Recent studies have identified the Rho guanine dissociation inhibitor, RhoGDI, as a target substrate for ubiquitination by GRAIL. Ubiquitination stabilizes this inhibitor, which sequesters Rho-family GTPases away from the membrane, preventing their effects on the reorganization of the cytoskeleton [40].

A member of the Casitas B lymphoma (Cbl) family of proteins, Cbl-b, has also been shown to be expressed in anergic T cells and to participate in the maintenance of the anergic state [41]. Cbl-b is a RING finger-containing E3 ubiquitin ligase expressed in hematopoietic cells and is involved in negative regulation of the TCR and other signalling pathways [42,43]. As discussed above, Egr proteins have been shown to control Cbl-b expression in anergic T cells [34]. Previous studies have indicated that Cbl-b ubiquitinates the p85 subunit of phosphoinositide-3-kinase (PI3K). This modification affects p85 subcellular localization, reducing its recruitment to the immunological synapse and, therefore, preventing the interaction of PI3K with CD28 and the TCR ζ chain [44]. Cbl-b can also regulate Vav1 activity, determining the phosphorylation state of this adaptor molecule and its recruitment to the immunological synapse [45]. Studies on Cbl-b-deficient mice indicate that this protein plays a key role in peripheral tolerance, as T cells from these mice, which develop autoimmunity, are resistant to anergy induction *in vivo* and *in vitro* and show reduced inactivation of PLC-γ1 in response to anergizing stimuli [41,46].

Itch is an 863-amino acid cytosolic protein containing a HECT homology domain that confers E3 ubiquitin ligase activity. Similar to Cbl-b and GRAIL, Itch expression is also upregulated during anergy induction in a calcium-/calcineurin-/NFAT-dependent manner [46]. In response to anergizing stimuli, Itch is rapidly upregulated at the mRNA and protein levels. When anergic T cells are re-stimulated, Itch localizes in

detergent-insoluble membrane microdomains where it mono-ubiquitinates PLC-γ and protein kinase C θ, promoting lysosomal degradation of these two enzymes. Degradation of PLC-γ and protein kinase C θ leads to a blockade of calcium flux and disrupts key signalling pathways that are responsible for the formation of the immunological synapse [46]. In addition, it has been also shown that Jun is a substrate of Itch, suggesting another level of regulation of the expression of IL-2 [47]. Supporting the role of Itch in the induction of peripheral tolerance, Itch-deficient mice develop a severe systemic lymphoproliferative disease, characterized by large secondary lymph node organs, chronic inflammation and itchy skin [48].

The expression of the diacylglycerol kinase-α (DGK-α) is also upregulated at the mRNA and protein levels during T cell anergy induction [27,46,49]. DGK-α phosphorylates DAG, which is converted to phosphatidic acid. Depletion of available DAG prevents the recruitment of the guanine nucleotide exchange factor RasGRP1 and results in the uncoupling of Ras activation from TCR engagement [49,50]. The role of DGK-α in the maintenance of T cell anergy is supported by the fact that inhibiting DGK-α using a pharmacological inhibitor can restore IL-2 production in anergic T cells [49]. Furthermore, mice deficient in DGK-α show hyper-responsive T cells *in vitro* and are significantly resistant to anergy induction both *in vitro* and *in vivo* [50].

Caspase 3 expression is also upregulated both at the mRNA and protein levels during T cell anergy [27]. Recent data from our laboratory show that increased expression of this protease is followed by its activation in non-apoptotic anergic T cells. Once activated, Caspase 3 associates with the plasma membrane and is responsible for cleaving and inactivating GADS and Vav1 following reactivation of the TCR.

1.5 Transcriptional repression in T cell anergy: epigenetic modification of the *Il2* promoter

Initial molecular characterizations of anergic T cells identified a defect in the activation of the Ras/MAPK signalling pathway that could account for the lack of activation of AP-1 complexes, which would cause decreased IL-2 production in these cells [51,52]. As discussed above, different mechanisms involving a number of proteins expressed in anergic T cells have been proposed to account for dampening TCR signalling; this ultimately leads to defective IL-2 production and hypo-responsiveness. However, evidence suggests that there are also active mechanisms of transcriptional repression that directly inhibit cytokine transcription. Early studies identified regions in the *Il2* promoter that seemed to be required for downregulating the activity of plasmids containing this promoter when transfected into anergic T cell clones [53,54]. Binding of cAMP-response-element-binding protein (CREB)–CREM (a splice variant which lacks the transactivation domain) complexes to that region was described, suggesting that isoforms of CREM, such as ICER, could form repressor complexes and inactivate

the *Il2* promoter [55,56]. In addition, other repressor complexes consisting of BCL-3 and nuclear factor κB 1 (NF-κB1) homodimers have also been shown to bind to the *Il2* promoter in a model of superantigen-induced T cell anergy [57].

Suppression of *Il2* transcription by Smad3 has also been suggested to be involved in the inhibition of the expression of this cytokine in tolerant T cells. Smad3 had previously been shown to mediate transforming growth factor β (TGF-β)-induced inhibition of IL-2 expression in T cells [58]. Experiments performed using an *in vivo* model of T cell tolerance induced by the blockade of CD40L and B7 proteins showed recently that the inability of tolerant T cells to downregulate the levels of $p27^{kip}$ in response to TCR engagement prevented proper activation of cyclin-dependent kinases needed to efficiently inactivate Smad3, causing inhibition of *Il2* transcription in those T cells. Supporting this mechanism, it was also reported that knocking-down Smad3 expression made T cells resistant to tolerizing stimuli [59].

In T cells the expression of the *Il2* gene is controlled by active chromatin remodelling of its locus. In naïve T cells, the distal *Il2* promoter shows a partially open conformation that allows low-level expression of this cytokine [60]. Following T cell activation, the *Il2* promoter is further remodelled, resulting in increased susceptibility to nucleases and increased levels of histone acetylation. This increase in histone acetylation at the *Il2* promoter, which requires signalling induced by CD28 engagement, allows for higher levels of expression of this cytokine in effector T cells [61]. We and others have recently shown that active mechanisms of transcriptional repression of the *Il2* gene occur in anergic T cells as a consequence of epigenetic modifications on the *Il2* promoter [28, 29]. Tolerizing stimuli induce these modifications that cause stable inhibition of *Il2* transcription. In response to partial stimulation, Ikaros expression is upregulated during induction of T cell anergy [28]. Ikaros is a member of a family of transcription factors critical for lymphoid development. This protein is also expressed in peripheral T cells, where it may play a role in the regulation of T cell activation-induced epigenetic modifications, helping to maintain the requirement of co-stimulatory signals to induce *Il2* expression [29]. Ikaros mediates transcriptional repression in anergic T cells by directly binding to the *Il2* promoter and recruiting histone deacetylases (HDACs). The subsequent deacetylation of histones 3 (H3) and 4 (H4) maintains the *Il2* promoter locus in a closed state and prevents transcription of this cytokine [28]. Activation of *Il2* transcription has also been associated with changes in the levels of DNA methylation of the *Il2* promoter, as TCR engagement leads to active demethylation of the *Il2* promoter/enhancer region [62]. Recently, an *in vivo* model of superantigen-induced T cell anergy demonstrated that decreased production of IL-2 and interferon-γ in anergic T cells correlated with increased levels of DNA methylation in these genes when compared with effector T cells [30].

Together the available data suggest that stimuli that induce T cell anergy would lead to the engagement of an active programme of epigenetic modifications that would result in the generation of a population of T cells in which a specific cytokine-expression pattern is epigenetically imprinted.

1.6 Regulatory T cells

Suppression by regulatory T cells (Tregs) is a mechanism of peripheral tolerance that exists to prevent self-reactive lymphocytes from initiating a harmful autoimmune response. Treg functions, however, are not limited to suppressing self-reactive T cells, as they are also implicated in regulating normal immune responses against pathogens, allografts and cancer cells [4,63–65]. Tregs were first described in the early 1970s and were thought to be a specific subset of T cells that could suppress T cell activation through secreted-antigen factors [66]. The identification of CD25 (IL-2 receptor α-chain) as a marker of suppressive $CD4^+$ T cells made it possible for the first time to monitor these cells [67]. With the utilization of CD25 as a marker, successive experiments revealed that transferring $CD25^+$ cells from adult mice was effective in preventing autoimmune disease. Several subtypes of Tregs have been characterized and the underlying suppressive mechanisms of each subtype are subjects of both intense research and controversy in the field. Whereas naturally occurring $CD4^+$ $CD25^+$ Tregs develop in the thymus and constitute approximately 2–10% of the total $CD4^+$ T cells, other populations of T cells with regulatory activity can also be generated in the periphery [68,69]. The discovery of Treg subtypes has provided insight into the critical roles these cells play in the proper maintenance of immune homeostasis. However, many questions about the biochemical and molecular basis of Treg function have yet to be answered. A clear understanding of how Tregs, both natural and adaptive, develop and confer suppression is critical in the development of novel therapies against autoimmune diseases.

1.7 Transcriptional control of Treg development and function

The transcription factor Foxp3, encoded on the X chromosome, is selectively expressed in Tregs and is required for their development in the thymus and for maintaining their regulatory function in the periphery [70–72]. The signals that regulate the expression of this transcription factor in Tregs are not yet defined clearly. Recent evidence has uncovered that the expression of Foxp3 is established in Tregs by stable epigenetic modifications on the *Foxp3* promoter region. In Tregs, this region shows CpG demethylation and histone modifications associated with active transcription, including H3 acetylation and H3 trimethylation at lysine 4 [73]. These changes are imprinted during Treg development in the thymus. Experiments performed in EL4 cells have also revealed that the *Foxp3* promoter contains a conserved enhancer element which shows a high level of H4 acetylation in Tregs and is synergistically activated by the transcription factors Smad3 and NFAT [74]. It has been suggested that Smad7, which is normally induced by TGF-β and negatively regulates TGF-β signalling, is down-regulated by Foxp3. The downregulation of Smad7 by Foxp3 should allow $CD25^-$ T cells to become highly sensitive to the ability of TGF-β to induce Treg development

via Smad3 and Smad4 [75]. Elements containing CREB-/activating transcription factor (ATF)- and NFAT-binding sites in enhancer or promoter regions of the *Foxp3* locus may also play a role in the regulation of Foxp3 expression in response to signals initiated by the engagement of the TCR [76,77].

Foxp3 is almost exclusively expressed in regulatory T cells and ectopic expression of Foxp3 in non-regulatory $CD4^+$ T cells induces the acquisition of suppressor activity [70,78]. Studies carried out in the last few years have provided data that clearly support the crucial role of Foxp3 as a Treg cell-specification factor. Mice deficient in Foxp3 do not develop $CD4^+CD25^+$ Treg populations [70]. Furthermore, deletion of Foxp3 in mature peripheral T cells leads to a loss in the suppressor activity of Tregs, indicating that this transcription factor is not only needed for Treg development, but its constant expression is required to maintain a Treg identity [71]. Two recent studies have begun to elucidate the programme of gene expression activated by Foxp3 in Tregs by comparing the gene-expression profiles of non-regulatory and regulatory T cells or from T cell hybridomas that either express or lack expression of Foxp3 [79,80]. Results from these studies have identified that, among the genes whose expression is regulated by Foxp3, two major groups are prominent: those coding for proteins involved in the regulation of T cell activation and TCR signalling, and genes encoding proteins that regulate transcription, including several transcription factors and proteins involved in chromatin modification. Although in many of these target genes Foxp3 acts as a transcriptional repressor, these analyses have also established that Foxp3 is able to directly or indirectly activate the expression of several genes. Chromatin immunoprecipitation experiments using anti-Foxp3 antibodies have also revealed that, although Foxp3 can bind to the promoters of some of those genes, the regulation of many Foxp3-targeted genes is not mediated directly by this transcription factor. The ability of Foxp3 to regulate the expression of several transcription factors may underlie Foxp-3-mediated regulation of a Treg cell-specific programme of gene expression.

The Foxp3-induced programme of gene expression that controls Treg differentiation and function is established in thymocytes and maintained in peripheral Tregs by epigenetic changes in Foxp3 target genes (Figure 1.3). Early reports have already identified that Tregs fail to remodel the chromatin at the *Il2* promoter, retaining it in a closed state in accordance with their anergic phenotype [81]. This effect has been recently reported to be a consequence of the recruitment of chromatin-modifying enzymes, linking promoter occupation by Foxp3 to histone deacetylation and suppression of transcription [80,82,83]. Ectopic expression of Foxp3 induces H3 deacetylation and suppression of the *Il2* and *Ifng* genes, whereas increased H3 acetylation and activation can be detected in the *Gitr*, *Il2ra* and *Ctla4* promoters. Foxp3 forms part of a complex that includes the histone deacetylases HDAC7 and 9 and the histone acetyl transferase TIP60, which are required for activating or suppressing the transcription of genes regulated by Foxp3 [82,84]. Foxp3 itself may also be regulated by acetylation, as TIP60-mediated acetylation of several lysine residues located in the fork domain activates Foxp3 and enhances binding to the *Il2* promoter [84].

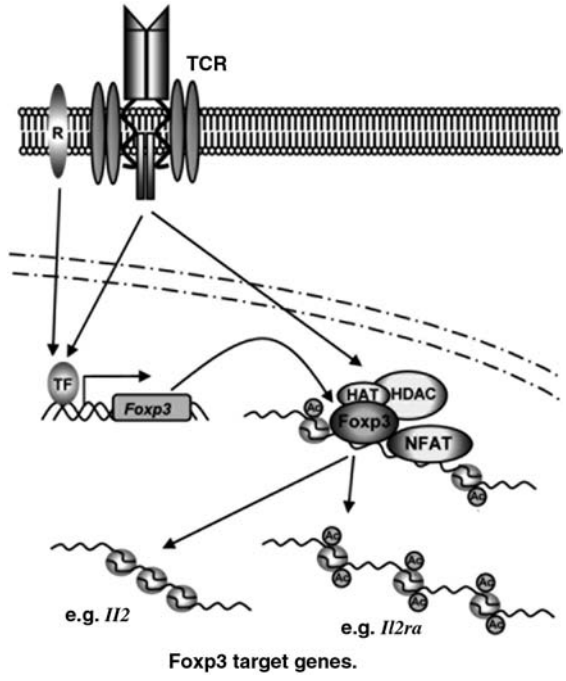

Figure 1.3 Foxp3 regulates transcription by direct occupancy of promoter regions and induction of epigenetic changes in its target genes. Foxp3 expression regulates Treg differentiation and is required to maintain a suppressor phenotype in peripheral Tregs. In the thymus, expression of Foxp3 is regulated by TCR engagement, and other receptors (R) such as CD28 have also been shown to play a role in inducing Foxp3 expression. TCR signalling is also required to express Foxp3 in peripheral T cells, with the likely contribution resulting from engagement of the TGF-β receptor. Foxp3 has been shown to bind in activation-dependent and -independent manners to specific target genes, where it has been shown to cooperate and form complexes with NFAT1. Foxp3 forms on the promoters of these target genes higher-order complexes by recruitment of chromatin-remodelling complexes that contain histone acetyl transferases (HATs) (Tip60) and HDACs class I and II. Activation of the expression of Foxp3-dependent genes, such as *Il2ra*, *Gitr* or *Ctla4*, is associated with increased histone acetylation, whereas suppression of the expression of genes, such as *Il2*, correlates with histone deacetylation. Foxp3 is also a substrate for those enzymes, and its activity is regulated by acetylation (Ac) of several residues in the fork domain.

The nature of the different transcriptional complexes that Foxp3 may form remains to be fully elucidated. It has been recently shown that Foxp3 may control Treg function through cooperation with NFAT to induce the upregulation of *Il2ra*, *Ctla4* and *Gitr*, and repress the expression of *Il2* [85]. Foxp3-mediated repression of NFAT-dependent transcription of *Il2* occurs after Foxp3 binds to a consensus sequence that overlaps with the AP-1 site, thereby suggesting that Foxp3 may compete with NFAT–AP-1 complexes on *Il2* regulatory elements. This claim has been corroborated by studies that resolved the crystal structure of a complex formed by the DNA binding domain of NFAT1 and the fork domain of Foxp2, which indicate cooperative binding of NFAT and Foxp3 to

DNA [85]. NFAT1 and Foxp3 can occupy the *Il2*, *Ctla4*, and *Il2ra* promoters, both in Tregs and in T cells transduced with a retrovirus directing the expression of Foxp3. Furthermore, expression of a Foxp3 form that contains a mutation that prevents interaction with NFAT fails to induce a suppressive phenotype, supporting a crucial role of these complexes in the differentiation of Treg cells. Therefore, NFAT proteins reveal themselves as key regulators of T cell fate by integrating signalling initiated at the TCR with signals resulting from engagement of other receptors such as CD28 or the TGF-β receptor. The availability of different transcriptional partners resulting from the activation of specific signalling pathways would determine whether T cells become activated (if NFAT cooperates with AP-1), differentiate into Tregs (when NFAT cooperates with Foxp3) or turn anergic (in the absence of other transcriptional partners).

Transcriptional mechanisms may not only regulate Treg differentiation but may also underlie the suppression of T cell activation by Tregs. Studies have demonstrated that T cells deficient in two members of the NFAT family of transcription factors, NFAT1 and NFAT4, cannot be suppressed by competent Tregs. This combined NFAT deficiency allows Tregs to develop normally but is still be able to cause an autoimmune phenotype [86,87]. Thus, it is likely that the presence of NFAT may be required in cells targeted for suppression by Tregs, where it could direct the expression of a specific set of genes that might be able to suppress T cell activation.

References

1. Sprent, J. and Kishimoto, H. (2002) The thymus and negative selection. *Immunological Reviews* **185**, 126–135.
2. Li, L.Q. and Boussiotis, V.A. (2006) Physiologic regulation of central and peripheral T cell tolerance: lessons for therapeutic applications. *Journal of Molecular Medicine* **84**, 887–899.
3. Macian, F., Im, S.H., Garcia-Cozar, F.J. *et al.* (2004) T-cell anergy. *Current Opinions in Immunology* **16**, 209–216.
4. Sakaguchi, S., Ono, M., Setoguchi, R. *et al.* (2006) Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunological Reviews* **212**, 8–27.
5. Schwartz, R.H. (2003) T cell anergy. *Annual Review of Immunology* **21**, 305–334.
6. Jenkins, M.K., Pardoll, D.M., Mizuguchi, J. *et al.* (1987) Molecular events in the induction of a nonresponsive state in interleukin 2-producing helper T-lymphocyte clones. *Proceedings of the National Academy of Sciences, USA* **84**, 5409–5413.
7. Quill, H. and Schwartz, R.H. (1987) Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. *Journal of Immunology* **138**, 3704–3712.
8. Mueller, D.L., Jenkins, M.K. and Schwartz, R.H. (1989) An accessory cell-derived costimulatory signal acts independently of protein kinase C activation to allow T cell proliferation and prevent the induction of unresponsiveness. *Journal of Immunology* **142**, 2617–2628.
9. Jenkins, M.K., Chen, C.A., Jung, G. *et al.* (1990) Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody. *Journal of Immunology* **144**, 16–22.
10. Korb, L.C., Mirshahidi, S., Ramyar, K. *et al.* (1999) Induction of T cell anergy by low numbers of agonist ligands. *Journal of Immunology* **162**, 6401–6409.

11. Sloan-Lancaster, J., Evavold, B.D. and Allen, P.M. (1993) Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. *Nature* **363**, 156–159.
12. Choi, S. and Schwartz, R.H. (2007) Molecular mechanisms for adaptive tolerance and other T cell anergy models. *Seminars in Immunology* **19**, 140–152.
13. Singh, N.J. and Schwartz, R.H. (2003) The strength of persistent antigenic stimulation modulates adaptive tolerance in peripheral CD4+ T cells. *Journal of Experimental Medicine* **198**, 1107–1117.
14. Perez, V.L., VanParijs, L., Biuckians, A. *et al.* (1997) Induction of peripheral T cell tolerance in vivo requires CTLA-4 engagement. *Immunity* **6**, 411–417.
15. Greenwald, R.J., Boussiotis, V.A., Lorsbach, R.B. *et al.* (2001) CTLA-4 regulates induction of anergy in vivo. *Immunity* **14**, 145–155.
16. Zarek, P.E., Huang, C.T., Lutz, E.R. *et al.* (2008) A2A receptor signaling promotes peripheral tolerance by inducing T-cell anergy and the generation of adaptive regulatory T cells. *Blood* **111**, 251–259.
17. Keir, M.E. and Sharpe, A.H. (2005) The B7/CD28 costimulatory family in autoimmunity. *Immunological Reviews* **204**, 128–143.
18. Dustin, M.L. (2005) A dynamic view of the immunological synapse. *Seminars in Immunology* **17**, 400–410.
19. Feske, S., Okamura, H., Hogan, P.G. *et al.* (2003) Ca2+/calcineurin signalling in cells of the immune system. *Biochemical and Biophysical Research Communications* **311**, 1117–1132.
20. Macian, F. (2005) NFAT proteins: Key regulators of T-cell development and function. *Nature Reviews Immunology* **5**, 472–484.
21. Crabtree, G.R. and Olson, E.N. (2002) NFAT signaling: choreographing the social lives of cells. *Cell* **109** (Suppl), S67–S79.
22. Rao, A., Luo, C. and Hogan, P.G. (1997) Transcription factors of the NFAT family: regulation and function. *Annual Review of Immunology* **15**, 707–747.
23. Hogan, P.G., Chen, L., Nardone, J. *et al.* (2003) Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes and Development* **17**, 2205–2232.
24. Lopez-Rodriguez, C., Aramburu, J., Rakeman, A.S. *et al.* (1999) NFAT5, a constitutively nuclear NFAT protein that does not cooperate with Fos and Jun. *Proceedings of the National Academy of Sciences, USA* **96**, 7214–7219.
25. Jain, J., McCaffrey, P.G., Valge-Archer, V.E. *et al.* (1992) Nuclear factor of activated T cells contains Fos and Jun. *Nature* **356**, 801–804.
26. Macian, F., Lopez-Rodriguez, C. and Rao, A. (2001) Partners in transcription: NFAT and AP-1. *Oncogene* **20**, 2476–2489.
27. Macian, F., Garcia-Cozar, F., Im, S.H. *et al.* (2002) Transcriptional mechanisms underlying lymphocyte tolerance. *Cell* **109**, 719–731.
28. Bandyopadhyay, S., Dure, M., Paroder, M. *et al.* (2007) Interleukin 2 gene transcription is regulated by Ikaros-induced changes in histone acetylation in anergic T cells. *Blood* **109**, 2878–2886.
29. Thomas, R.M., Chunder, N., Chen, C. *et al.* (2007) Ikaros enforces the costimulatory requirement for IL2 gene expression and is required for anergy induction in CD4+ T lymphocytes. *Journal of Immunology* **179**, 7305–7315.
30. Thomas, R.M., Saouaf, S.J. and Wells, A.D. (2007) Superantigen-induced CD4+ T cell tolerance is associated with DNA methylation and histone hypo-acetylation at cytokine gene loci. *Genes and Immunity* **8**, 613–618.
31. Lechner, O., Lauber, J., Franzke, A. *et al.* (2001) Fingerprints of anergic T cells. *Current Biology* **11**, 587–595.
32. Giffin, M.J., Stroud, J.C., Bates, D.L. *et al.* (2003) Structure of NFAT1 bound as a dimer to the HIV-1 LTR kappa B element. *Nature Structural Biology* **10**, 800–806.

33. Jin, L., Sliz, P., Chen, L. *et al.* (2003) An asymmetric NFAT1 dimer on a pseudo-palindromic kappa B-like DNA site. *Nature Structural Biology* **10**, 807–811.
34. Safford, M., Collins, S., Lutz, M.A. *et al.* (2005) Egr-2 and Egr-3 are negative regulators of T cell activation. *Nature Immunology* **6**, 472–480.
35. Fathman, C.G. and Lineberry, N.B. (2007) Molecular mechanisms of CD4(+) T-cell anergy. *Nature Reviews Immunology* **7**, 599–609.
36. Heissmeyer, V., Macian, F., Varma, R. *et al.* (2005) A molecular dissection of lymphocyte unresponsiveness induced by sustained calcium signalling. *Novartis Foundation Symposium* **267**, 165–174.
37. Anandasabapathy, N., Ford, G.S., Bloom, D. *et al.* (2003) GRAIL: an E3 ubiquitin ligase that inhibits cytokine gene transcription is expressed in anergic CD4+ T cells. *Immunity* **18**, 535–547.
38. Soares, L., Seroogy, C., Skrenta, H. *et al.* (2004) Two isoforms of otubain 1 regulate T cell anergy via GRAIL. *Nature Immunology* **5**, 45–54.
39. Seroogy, C.M., Soares, L., Ranheim, E.A. *et al.* (2004) The gene related to anergy in lymphocytes, an E3 ubiquitin ligase, is necessary for anergy induction in CD4 T cells. *Journal of Immunology* **173**, 79–85.
40. Su, L., Lineberry, N., Huh, Y. *et al.* (2006) A novel E3 ubiquitin ligase substrate screen identifies Rho guanine dissociation inhibitor as a substrate of gene related to anergy in lymphocytes. *Journal of Immunology* **177**, 7559–7566.
41. Jeon, M.S., Atfield, A., Venuprasad, K. *et al.* (2004) Essential role of the E3 ubiquitin ligase Cbl-b in T cell anergy induction. *Immunity* **21**, 167–177.
42. Bachmaier, K., Krawczyk, C., Kozieradzki, I. *et al.* (2000) Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature* **403**, 211–216.
43. Chiang, Y.J., Kole, H.K., Brown, K. *et al.* (2000) Cbl-b regulates the CD28 dependence of T-cell activation. *Nature* **403**, 216–220.
44. Fang, D. and Liu, Y.C. (2001) Proteolysis-independent regulation of PI3K by Cbl-b-mediated ubiquitination in T cells. *Nature Immunology* **2**, 870–875.
45. Krawczyk, C., Bachmaier, K., Sasaki, T. *et al.* (2000) Cbl-b is a negative regulator of receptor clustering and raft aggregation in T cells. *Immunity* **13**, 463–473.
46. Heissmeyer, V., Macian, F., Im, S.H. *et al.* (2004) Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins. *Nature Immunology* **5**, 255–265.
47. Gao, E.K., Lo, D., Cheney, R. *et al.* (1988) Abnormal differentiation of thymocytes in mice treated with cyclosporin A. *Nature* **336**, 176–179.
48. Perry, W.L., Hustad, C.M., Swing, D.A. *et al.* (1998) The itchy locus encodes a novel ubiquitin protein ligase that is disrupted in a18H mice. *Nature Genetics* **18**, 143–146.
49. Zha, Y.Y., Marks, R., Ho, A.W. *et al.* (2006) T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase-alpha. *Nature Immunology* **7**, 1166–1173.
50. Olenchock, B.A., Guo, R., Carpenter, J.H. *et al.* (2006) Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. *Nature Immunology* **7**, 1174–1181.
51. Fields, P.E., Gajewski, T.F. and Fitch, F.W. (1996) Blocked ras activation in anergic CD4(+) T cells. *Science* **271**, 1276–1278.
52. Li, W., Whaley, C.D., Mondino, A. *et al.* (1996) Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4(+) T cells. *Science* **271**, 1272–1276.
53. Kitagawa-Sakakida, S. and Schwartz, R.H. (1996) Multifactor cis-dominant negative regulation of IL-2 gene expression in anergized T cells. *Journal of Immunology* **157**, 2328–2339.
54. Becker, J.C., Brabletz, T., Kirchner, T. *et al.* (1995) Negative transcriptional regulation in anergic T cells. *Proceedings of the National Academy of Sciences, USA* **92**, 2375–2378.
55. Powell, J.D., Lerner, C.G., Ewoldt, G.R. *et al.* (1999) The -180 site of the IL-2 promoter is the target of CREB/CREM binding in T cell anergy. *Journal of Immunology* **163**, 6631–6639.

56. Bodor, J., Feigenbaum, L., Bodorova, J. *et al.* (2001) Suppression of T-cell responsiveness by inducible cAMP early repressor (ICER). *Journal of Leukocyte Biology* **69**, 1053–1059.
57. Grundstrom, S., Anderson, P., Scheipers, P. *et al.* (2004) Bcl-3 and NFkappaB p50-p50 homodimers act as transcriptional repressors in tolerant CD4+ T cells. *Journal of Biological Chemistry* **279**, 8460–8468.
58. McKarns, S.C., Schwartz, R.H. and Kaminski, N.E. (2004) Smad3 is essential for TGF-beta 1 to suppress IL-2 production and TCR-induced proliferation, but not IL-2-induced proliferation. *Journal of Immunology* **172**, 4275–4284.
59. Li, L., Iwamoto, Y., Berezovskaya, A. *et al.* (2006) A pathway regulated by cell cycle inhibitor p27 (Kip1) and checkpoint inhibitor Smad3 is involved in the induction of T cell tolerance. *Nature Immunology* **7**, 1157–1165.
60. Ward, S.B., Hernandez-Hoyos, G., Chen, F. *et al.* (1998) Chromatin remodeling of the interleukin-2 gene: distinct alterations in the proximal versus distal enhancer regions. *Nucleic Acids Research* **26**, 2923–2934.
61. Thomas, R.M., Gao, L. and Wells, A.D. (2005) Signals from CD28 induce stable epigenetic modification of the IL-2 promoter. *Journal of Immunology* **174**, 4639–4646.
62. Bruniquel, D. and Schwartz, R.H. (2003) Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. *Nature Immunology* **4**, 235–240.
63. Zou, W. (2006) Regulatory T cells, tumour immunity and immunotherapy. *Nature Reviews Immunology* **6**, 295–307.
64. Baecher-Allan, C. and Hafler, D.A. (2006) Human regulatory T cells and their role in autoimmune disease. *Immunological Reviews* **212**, 203–216.
65. Bharat, A., Fields, R.C. and Mohanakumar, T. (2005) Regulatory T cell-mediated transplantation tolerance. *Immunology Research* **33**, 195–212.
66. Gershon, R.K. and Kondo, K. (1970) Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology* **18**, 723–737.
67. Sakaguchi, S., Sakaguchi, N., Asano, M. *et al.* (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *Journal of Immunology* **155**, 1151–1164.
68. Bluestone, J.A. and Abbas, A.K. (2003) Natural versus adaptive regulatory T cells. *Nature Reviews Immunology* **3**, 253–257.
69. Kronenberg, M. and Rudensky, A. (2005) Regulation of immunity by self-reactive T cells. *Nature* **435**, 598–604.
70. Fontenot, J.D., Gavin, M.A. and Rudensky, A.Y. (2003) Foxp3 programs the development and function of CD4+ CD25+ regulatory T cells. *Nature Immunology* **4**, 330–336.
71. Williams, L.M. and Rudensky, A.Y. (2007) Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nature Immunology* **8**, 277–284.
72. Zheng, Y. and Rudensky, A.Y. (2007) Foxp3 in control of the regulatory T cell lineage. *Nature Immunology* **8**, 457–462.
73. Floess, S., Freyer, J., Siewert, C. *et al.* (2007) Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biology* **5**, e38.
74. Tone, Y., Kojima, Y., Furuuchi, K. *et al.* (2007) OX40 gene expression is up-regulated by chromatin remodeling in its promoter region containing Sp1/Sp3, YY1, and NF-kappa B binding sites. *Journal of Immunology* **179**, 1760–1767.
75. Fantini, M.C., Becker, C., Monteleone, G. *et al.* (2004) Cutting edge: TGF-beta induces a regulatory phenotype in CD4(+)CD25(−) T cells through Foxp3 induction and down-regulation of Smad7. *Journal of Immunology* **172**, 5149–5153.

76. Kim, H.P. and Leonard, W.J. (2007) CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. *Journal of Experimental Medicine* **204**, 1543–1551.
77. Mantel, P.Y., Ouaked, N., Ruckert, B. *et al.* (2006) Molecular mechanisms underlying FOXP3 induction in human T cells. *Journal of Immunology* **176**, 3593–3602.
78. Hori, S., Nomura, T. and Sakaguchi, S. (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057–1061.
79. Marson, A., Kretschmer, K., Frampton, G.M. *et al.* (2007) Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature* **445**, 931–935.
80. Zheng, Y., Josefowicz, S.Z., Kas, A. *et al.* (2007) Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature* **445**, 936–940.
81. Su, L., Creusot, R.J., Gallo, E.M. *et al.* (2004) Murine CD4+ CD25+ regulatory T cells fail to undergo chromatin remodeling across the proximal promoter region of the IL-2 gene. *Journal of Immunology* **173**, 4994–5001.
82. Chen, C., Rowell, E.A., Thomas, R.M. *et al.* (2006) Transcriptional regulation by Foxp3 is associated with direct promoter occupancy and modulation of histone acetylation. *Journal of Biological Chemistry* **281**, 36828–36834.
83. Li, B., Samanta, A., Song, X. *et al.* (2007) FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. *Proceedings of the National Academy of Sciences, USA* **104**, 4571–4576.
84. Tao, R., de Zoeten, E.F., Ozkaynak, E. *et al.* (2007) Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nature Medicine* **13**, 1299–1307.
85. Wu, Y., Borde, M., Heissmeyer, V. *et al.* (2006) FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* **126**, 375–387.
86. Ranger, A.M., Oukka, M., Rengarajan, J. *et al.* (1998) Inhibitory function of two NFAT family members in lymphoid homeostasis and Th2 development. *Immunity* **9**, 627–635.
87. Bopp, T., Palmetshofer, A., Serfling, E. *et al.* (2005) NFATc2 and NFATc3 transcription factors play a crucial role in suppression of CD4 + T lymphocytes by CD4 + CD25 + regulatory T cells. *Journal of Experimental Medicine* **201**, 181–187.

Abstract

Self-reactive T cells that escape negative selection in the thymus must be kept under control in the periphery. Intrinsic functional inactivation and mechanisms of dominant tolerance mediated by regulatory T cells exist to keep self-reactive T cells in check. Both systems are regulated by the activation of specific programmes of gene expression. Suboptimal stimulation of T cells results in the activation of a calcium-/nuclear factor of activated T cells (NFAT)-dependent cell-intrinsic programme of inactivation that, among other consequences, induces epigenetic changes in the interleukin (IL)-2 promoter that silence IL-2 expression and make T cells hypo-responsive to subsequent stimulation. Foxp3 directs the expression of set of genes that specify regulatory T cell development. In regulatory T cells, Foxp3 mediates activation and suppression of transcription of target genes by recruiting a multi-subunit complex involved in histone modification and chromatin remodelling.

Key words: anergy; regulatory T cell; NFAT; Ikaros; Foxp3; interleukin 2; histone deacetylase; histone acetyl transferase

2

Epigenetic regulation of Foxp3 expression in regulatory T cells

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2.1 Introduction

The immune system is highly effective in protecting us from microbial infections and tumour formation. However, it must be strictly controlled to prevent collateral tissue damage in exuberant immune reactions as well as accidental aggression against healthy tissue of the body by self-reactive immune cells. For this, the immune system must be able to discriminate between harmful non-self-antigens and harmless self-antigens, and ensure self-tolerance while maintaining protective immunity against invading pathogens. To this end, various mechanisms of self-tolerance have evolved which either eliminate lymphocytes that happen to express autoreactive antigen receptors during development and maturation – referred to as central tolerance – or inactivate and/or delete in the periphery those mature self-reactive cells that have managed to escape central tolerance mechanisms: so-called peripheral tolerance. Besides peripheral tolerance mechanisms such as induction of T cell anergy, clonal deletion and immunological ignorance, a dominant suppression mechanism mediated by some kind of 'suppressor T cell' has long been postulated [1,2]. However, since initial studies on this topic were difficult to reproduce, mainly due to technical hurdles, the suppressor-cell concept soon lost credibility [3]. In 1995 the idea was revived, when Sakaguchi *et al.* described a subpopulation of $CD4^+$ T cells that was able to prevent autoimmune disease driven by adoptive transfer of T cells into nude mice [4]. With this first

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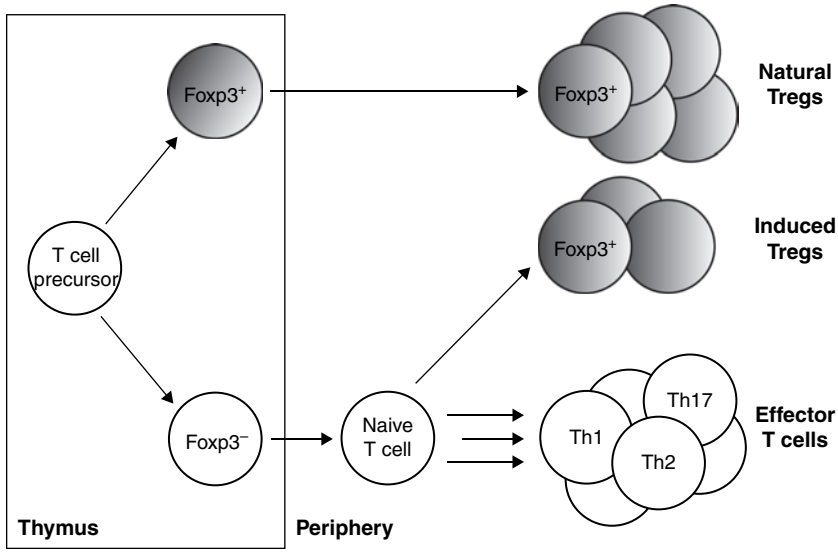

Figure 2.1 Development of Foxp3$^+$CD4$^+$ Tregs. The majority of Foxp3$^+$CD4$^+$ Tregs are generated in the thymus as a separate lineage (natural Tregs). However, Foxp3 expression can also be induced in the periphery from naïve Foxp3$^-$CD4$^+$ T cells upon activation under tolerogenic conditions (induced Tregs). Their percentage in the peripheral Treg pool is currently unclear, as is their stability.

characterization of a 'suppressor' T cell population, which was renamed regulatory T cells, the hypothesis of a suppression mechanism, which is dominant and can be transferred from one individual to another, was proven. Three years later, Thornton and Shevach established an *in vitro* suppression assay system by which the suppressive capacity of regulatory T cells could be easily tested [5]. With these tools at hand, the field of regulatory T cell research was opened and has led to the identification of multiple populations that share the characteristic suppressive activity but differ in their phenotypes, mode of action, route of generation and anatomical location [6]. By far the best-characterized suppressor cells are the naturally occurring Foxp3$^+$CD25$^+$CD4$^+$ regulatory T cells (Tregs), which are mainly generated in the thymus as a separate lineage (Figure 2.1).

2.2 Naturally occurring CD25$^+$ CD4$^+$ Tregs

Role(s) in the immune system

Tregs have been shown to possess a protective function in multiple autoimmune settings such as type 1 diabetes, multiple sclerosis, rheumatoid arthritis and inflammatory bowel disease (reviewed in [7]). Tregs harbour a large therapeutic potential since they can be activated in an antigen-specific manner and might therefore suppress

autoimmune reactions while leaving protective immunity directed against pathogen-derived antigens unaffected. This feature makes them a promising target for cellular therapy strategies in clinical autoimmunity settings, which are currently treated by rather unspecific immunosuppressive drugs, with drastic side effects on the whole immune system. Although Tregs might be best known for their role in peripheral self-tolerance and thereby in the prevention of autoimmunity, this is definitely not their only function. Actually, it seems rather that Tregs are involved in many different types of immune reaction and therefore might also be exploited in other therapeutical approaches. For example, several studies have analysed the ability of Tregs to facilitate the acceptance of allo-grafts in solid-organ transplantation experiments by suppressing allo-reactive immune reactions in an antigen-specific manner (reviewed in [8]). Tregs have also been shown to dampen allergic responses and protocols for the *in vivo* induction of allergen-specific Tregs are currently being developed [9]. Even during protective immunity against pathogens Tregs play an important role [10], since they have been shown to keep pro-inflammatory immune responses in check to prevent collateral tissue damage and also to resolve the reaction once the pathogen has been successfully defeated. They even seem to contribute to the formation of an immunological memory as they slow down or even inhibit the complete clearance of the pathogen, thereby providing a prolonged antigen supply. In contrast, in certain infection models Tregs have also been attributed to be responsible for the development of chronicity, in which case the deletion or reduction of Tregs might be a useful therapeutic approach. This also seems to be the method of choice with regard to tumour immunity as tumour-associated antigens often are self-antigens and tumour-rejecting reactions are therefore suppressed by Tregs [11]. Overall, as implicated by their name, Tregs seem to regulate various (if not all) types of immune responses and therefore controlled modulation of their number, functionality or activation state could enhance or inhibit immune reactions as needed and bring the immune system back into balance.

Mode of action: suppression mechanisms

Tregs may interact and suppress various types of leucocytes. For non-regulatory T cells, suppression can be monitored by reduced proliferation of activated T cells in co-cultures with Tregs when compared with cultures without the suppressors. Moreover, suppressed T cells also fail to produce interleukin (IL)-2 [5]. Intense research has aimed at identifying the mechanism by which Tregs exert their suppressive function. It seems, however, that rather than one, there are multiple mechanisms used by Tregs to inhibit activation and/or effector functions of non-regulatory T cells, and probably also other leucocyte subsets (reviewed in [12]). This diversity of action mechanisms makes sense if one considers the variety of immune responses that Tregs are able to control. In this way, Tregs are well prepared to react to different situations and can 'choose' the mode of suppression that fits best according to the nature of the immune response, the anatomical site of action and the initiating antigen.

There are four general modes of suppression that have been identified so far. First, due to their activated phenotype, Tregs can better compete for survival factors and deprive their environment of certain cytokines which are essential for the activation of naïve T cells (e.g. IL-2), which then in turn cannot fully get activated and fail to proliferate or even die by apoptosis [13]. This mechanism requires close proximity of Tregs and their target cell. Indeed, when separated by a transwell in suppression assays *in vitro*, Tregs lose their ability to suppress [14]. Second, some mechanisms seem to require direct T cell–T cell interactions. For example, Tregs have high levels of cAMP, a second messenger known to inhibit activation and IL-2 production in T cells. As a mode of suppression it has been shown that Tregs are able to deliver cAMP via gap junctions to their target cell [15]. Third, Tregs can also modulate the expression of co-stimulatory molecules on antigen-presenting cells, which then are less potent in activating non-regulatory T cells [16]. In accordance with this theory, the inhibitory receptor cytotoxic T lymphocyte antigen 4 (CTLA-4), which is constitutively expressed on Tregs and can interact with B7 molecules on antigen-presenting cells, has been discussed as a crucial player for the suppressive activity of Tregs [17]. Finally, while dispensable *in vitro*, *in vivo* data suggests a role for inhibitory cytokines such as IL-10, transforming growth factor β (TGF-β) or IL-35 for Treg-mediated suppression under certain conditions [12]. Moreover, other Treg-derived soluble factors such as perforin or granzyme B might also contribute to Treg activity by direct killing of the responder cells [18,19]. Taken together, our current knowledge indicates that the mode of action of Tregs is manifold and that it will be difficult to delineate the suppression mechanisms crucial for Treg activity in a given situation.

Phenotype: Treg markers

In a healthy mouse 10 to 15% of the $CD4^+$ T cell pool constitutively expresses the α-chain of the IL-2 receptor (CD25) on the surface, a molecule that is usually only upregulated in response to activation through the T cell receptor (TCR). It was this constitutive expression of CD25 that Sakaguchi and coworkers identified as the first surface maker for the Treg population [4]. Although in the steady state CD25 is a useful marker for Tregs, it is difficult to distinguish them from recently activated non-regulatory T cells that might occur in large numbers during infection or under inflammatory conditions. This is especially problematic in humans, who are permanently exposed to infectious agents and therefore harbour a lot of CD25-expressing cells in the peripheral blood. Other activation markers, such as CTLA-4 [17], glucocorticoid-induced tumour necrosis factor receptor (GITR) [20] and OX-40 [21], have also been found to be constitutively expressed on Tregs, but they encounter the same limitations for the unambiguous identification of Tregs.

To date, still the most reliable marker for Treg identification is the forkhead box transcription factor Foxp3 (or scurfin; in the human system it is FOXP3), which will be discussed in detail in the next section. Although Foxp3 expression seems to be highly specific for the Treg subclass [22–24], its intracellular expression poses a profound

drawback for its use to isolate vital Tregs from heterogeneous populations. While this hurdle has been circumvented for research purposes in the murine system by the generation of Foxp3–reporter mouse strains [22,25–27], it remains unsolved for the human system. It was even exacerbated by the finding that transient FOXP3 expression is not exclusive for the Treg population [28] and does also occur in recently activated human non-regulatory T cells [29–32]. The lack of a Treg-specific surface marker pinpoints one major pothole on the way to the therapeutical applicability of human Tregs in clinical settings.

2.3 The transcription factor FOXP3: determining Treg function and identity

The *Foxp3* gene was identified when Brunkow *et al.* were seeking for the cause of the aggressive autoimmune disease that can be observed in the natural mouse mutant scurfy [33]. Male scurfy mice develop uncontrolled exuberant immune responses and succumb to a multi-organ lymphoproliferative disease early in life. They show a frame-shift mutation in exon 8 of the *Foxp3* gene (insertion of two additional adenosines), which causes a premature stop and truncation of the forkhead box region of the protein, resulting in a highly unstable and non-detectable Foxp3 protein. Since *Foxp3* is located on the X chromosome, only hemizygous male mice are affected. The same phenotype was observed when Foxp3-knockout mice were generated by targeted gene disruption, confirming that lack of Foxp3 was indeed the cause for disease [22,23]. Mutations in the *Foxp3* gene and absence of functional FOXP3 protein has also been found to be responsible for immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome, a rare but fatal autoimmune disorder in young male patients (reviewed in [34]). Symptoms include enteropathy, eczema, anemia, thrombocytopenia, type 1 diabetes and thyroiditis mediated by hyper-reactive T cells and therefore resemble the scurfy phenotype in mice. These findings suggest that mutations in or deletion of the *Foxp3* gene lead to the absence of functional Tregs and thereby to the breakdown of peripheral tolerance, manifesting in the onset of autoimmunity. Indeed, suppressive $CD25^+CD4^+$ Tregs were absent in scurfy-/*Foxp3*-knockout mice and these animals could be rescued by transfer of Foxp3-competent $CD25^+CD4^+$ T cells [22]. In two recent studies, it could be further confirmed that 'removal' of $Foxp3^+$ Tregs is indeed sufficient to induce a scurfy-like phenotype in formerly completely healthy animals [25,35].

The absence of suppressive Tregs in scurfy-/*Foxp3*-knockout mice led to the hypothesis that Foxp3 is a lineage-determination factor and a master switch for the generation of Tregs. However, more recent studies presented convincing data that some Treg characteristics develop even in the absence of functional Foxp3 [36,37], suggesting that other factors beside Foxp3 might switch on the Treg developmental programme. Nevertheless, when Foxp3 expression was shut off in fully mature peripheral Tregs, these cells lost most of their Treg characteristics [38]. Viewed as a whole, Foxp3 seems to act as a lineage-stabilization factor, maintaining the Treg

identity, which has been initially instructed by other, so far unidentified, signals. But Foxp3 is not only involved in Treg maintenance, it also imparts Treg function. This was shown by retrovirus-mediated over-expression of Foxp3, which conferred suppressive properties to non-regulatory $CD25^{-}CD4^{+}$ T cells [22–24]. *Vice versa*, peripheral Tregs, which were fully functional, lost their suppressive capacities when Foxp3 expression was experimentally shut off [36,38]. Moreover, when Foxp3 expression was induced transiently in murine T cells by stimulation in the presence of TGF-β, cells only displayed suppressive activity as long as Foxp3 expression was maintained [39]. Taken together these data identify Foxp3 as the key regulator for the suppressive function of Tregs.

On the molecular level, Foxp3 displays features of a *bona fide* transcription factor as it binds to DNA via its forkhead box DNA-binding domain [40] and regulates the level of transcription of its target genes [41–44]. Of note, binding to DNA is strictly dependent on TCR signalling [44]. Although lacking a characterized transactivation domain, Foxp3 not only acts as a transcriptional repressor, but on certain genes also as a transcriptional activator [41,42]. It has been shown to physically interact with other important T cell transcription factors such as the nuclear factor of activated T cells (NFAT) and the nuclear factor κB (NF-κB) and interferes with their ability to transactivate their target genes [43,45]. This mechanism is best understood for the *Il-2* gene, the expression of which in normal T cells is activated by cooperative binding of NFAT and activator protein-1 (AP-1) after TCR-mediated signalling. However, when Foxp3 is present it outcompetes AP-1 and binds together with NFAT to an overlapping site in the *Il-2* promoter, which results in the suppression of *Il-2* transcription [43]; this accounts for the failure of Tregs to produce IL-2 after activation. A rather large pool of Foxp3 target genes has already been mapped [41,42]. However, in what way these genes contribute to Treg physiology and function remains to be investigated.

2.4 Molecular regulation of FOXP3

The molecular regulation of Foxp3 expression has also been a field of intense research. So far, the human *FOXP3* promoter has been mapped to a region 6.5 kb upstream of the translational start site ([46]; for an overview of the *Foxp3* locus see Figure 2.2). Its activity is dependent on NFAT and AP-1, two transcription factors usually activated in response to TCR-mediated signalling. Binding of the T helper (Th) 2-lineage-specification factor GATA3 to the promoter has been reported to inhibit *Foxp3* transcription [47]. Along this line, the signal transducer and activator of transcription-1 (STAT1) seems to suppress Foxp3 induction in the presence of Th1-polarizing cytokines [48]. On the contrary, IL-2-mediated binding of STAT5 seems to promote Foxp3 expression [49,50]. In addition to the promoter, two other transcriptionally active enhancer elements have been found in the first intron of the *Foxp3* gene. Whereas one of them is bound by Smad3 and NFAT and thereby functions as a TGF-β-sensitive enhancer [51], the other seems to be involved in Foxp3 regulation on the epigenetic level, as will be discussed in the next section.

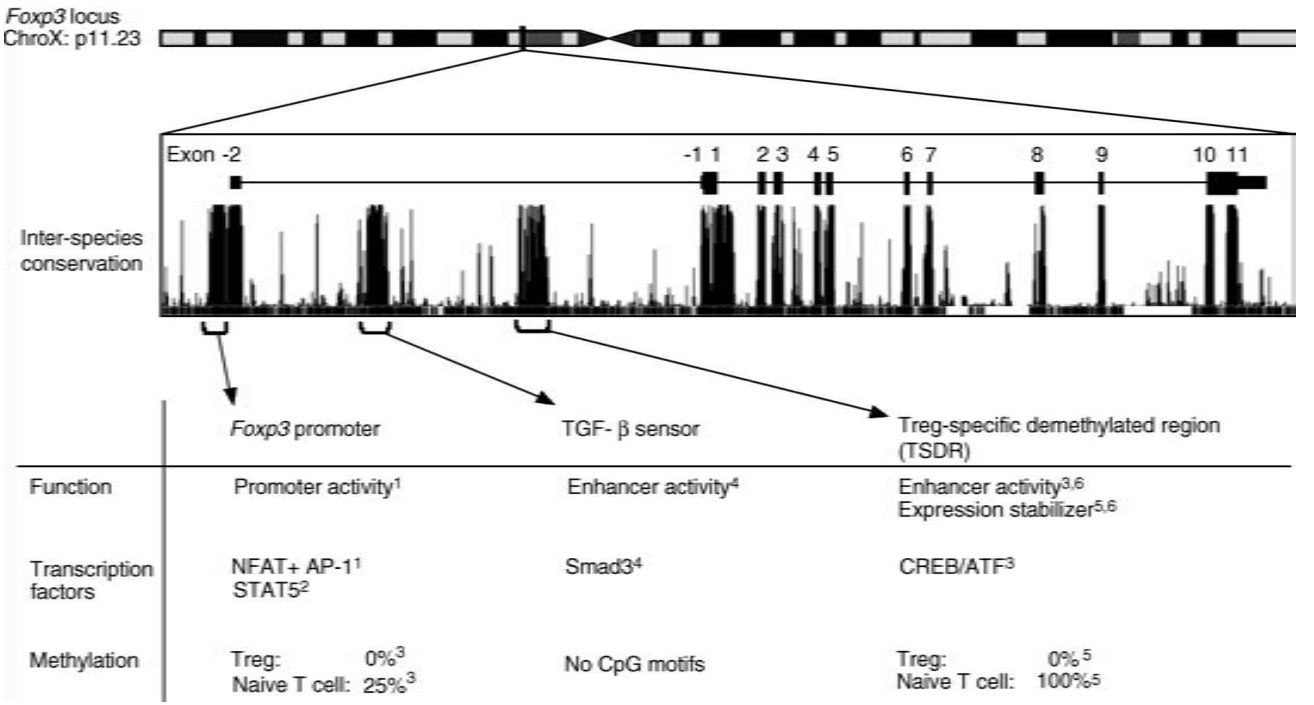

Figure 2.2 Schematic representation of the *Foxp3* locus. Within the inter-species conservation analysis three evolutionarily conserved non-coding elements are highlighted, which have been found to participate in the transcriptional regulation of *Foxp3* transcription. References: superscript numbers 1–6 denote the following references: 1, [46]; 2, [50]; 3, [55]; 4, [51]; 5, [39]; 6, [54]. CREB/ATF, cAMP-response-element-binding protein/activating transcription factor; STAT, signal transducer and activator of transcription.

2.5 Tregs as a stable lineage: indications of epigenetic imprinting

Purified *ex vivo* Foxp3$^+$ Tregs, which were expanded *in vitro* or were adoptively transferred into lymphopenic or fully immunocompetent recipient mice, displayed stable Foxp3 expression even after several rounds of cell division [39,52]. Considering that Foxp3 has been identified as the Treg lineage stabilization factor it is reasonable to suggest that expression of this transcription factor might be epigenetically imprinted to ensure a stable Treg pool in the periphery. Fixation of a certain differentiation state by epigenetic mechanisms has been shown to occur in other T cell subpopulations, such as Th1 and Th2 cells, where the expression of the characteristic cytokines interferon-γ and IL-4 get reciprocally imprinted at both gene loci [53]. Indeed, by *in silico* analysis we were able to identify an evolutionary conserved region in the first intron of the *Foxp3* gene, which was rich in CpG motifs, indicating an epigenetically active element.

On average, CpG motifs are under-represented in the DNA sequence of mammals because the cytosine of the CpG dinucleotide can get methylated by intracellular methylases and then is prone to inducing point mutations by accidental deamination, which turns a methylated (but not an unmethylated) cytosine into a thymidine. Therefore CpG motifs have only been preserved by evolution in regions where they have an important function. This is often the case in regions that participate in the regulation of gene expression. For this, CpG motifs have a special feature as they can occur in two states: methylated or demethylated. When methylated, they form a marker for methyl-DNA-binding proteins, which recruit transcriptional co-repressors to silence gene transcription at the surrounding locus and initiate condensation of the chromatin with low DNA accessibility. This 'closed' chromatin state that does not allow active transcription is usually associated with certain ('repressive') histone modifications. *Vice versa*, demethylated CpG motifs are indicative of an open and accessible chromatin state with 'permissive' histone modification and active transcription. Apart from this mechanism, methylation at CpG motifs can also interfere with the DNA-binding ability of certain transcription factors with recognition sequences that span such motifs. Most interestingly, DNA methylation patterns are copied and maintained during cell division, making methylation-regulated gene-expression patterns heritable from a cell to its daughter cells. Still, the system is flexible and can be modulated if needed as methylation marks can be added or removed upon appropriate signalling. CpG methylation patterns therefore represent a level of gene regulation that can be modulated but which is heritable at a given state and is independent of the DNA sequence. In addition, the methylation state of CpG-rich regions provides information about the activation state of the neighbouring gene loci.

When we analysed the methylation pattern of the conserved CpG-rich element in the *Foxp3* locus comparing murine *ex vivo* isolated Foxp3$^+$CD25$^+$CD4$^+$ Tregs with Foxp3$^-$CD25$^-$CD4$^+$ T cells, Tregs showed complete demethylation on all CpG motifs, whereas – on the contrary – Foxp3$^-$ T cells were fully methylated [39]. Such a differential methylation pattern could not be found at a CpG-rich control region in

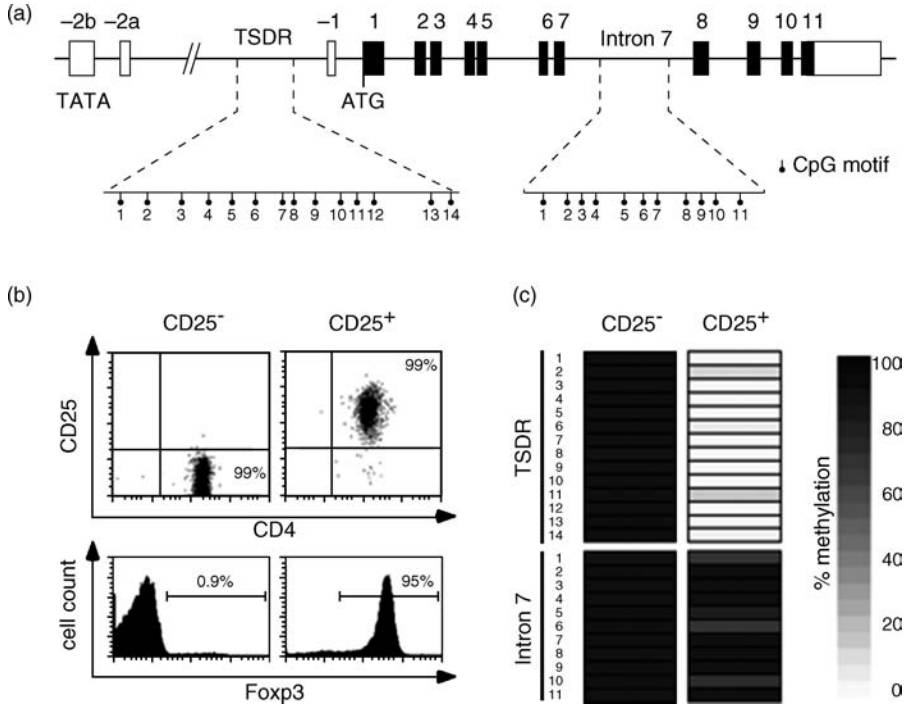

Figure 2.3 Foxp3$^+$CD25$^+$CD4$^+$ Tregs show a selectively demethylated state of the Treg-specific demethylated region (TSDR). (a) Schematic representation of the *Foxp3* locus and the regions analysed for their methylation status. (b) Phenotype of purified Foxp3$^-$CD25$^-$CD4$^+$ and Foxp3$^+$CD4$^+$CD25$^+$ T cells, which were isolated by FACS from lymphoid organs of BALB/c mice and used for methylation analysis. (c) Methylation status of the TSDR and a control region in intron 7 in Foxp3$^-$CD25$^-$CD4$^+$ and Foxp3$^+$CD4$^+$CD25$^+$ T cells. Each box represents an individual CpG motif. The degree of methylation is coded according to the shades of grey shown on the scale. Modified from [39].

intron 7 of the *Foxp3* gene, which was strongly methylated in both cell types, indicating that the selective demethylated state of Tregs in the evolutionarily conserved intron-1 element did not occur by chance (Figure 2.3). We therefore termed this element Treg-specific demethylated region (TSDR). As a further indication that TSDR is indeed involved in the epigenetic regulation of Foxp3 expression, we observed permissive histone modifications (acetylated histone 3 and 4; trimethylated lysine 4 at histone 3) in this region in Foxp3$^+$ Tregs but not in Foxp3$^-$ control cells. To test whether imprinting at TSDR occurs already during thymic development, we analysed CD25$^+$CD4SP thymocytes, which expressed Foxp3 to a high degree. However, the average demethylation of TSDR in these cells was only around 50%. Since methylation as a DNA modification occurs in a binary fashion with regard to a single CpG motif, this result shows that Foxp3$^+$ thymocytes are still heterogeneous with respect to TSDR demethylation and only a subfraction displays an already remodelled *Foxp3* locus. Moreover, it indicates that mere Foxp3 expression can also occur when TSDR is in a methylated state, a finding that we confirmed with TGF-β-induced Tregs (see below).

Using luciferase assays we could verify that TSDR indeed can act as a transcriptional enhancer for the *Foxp3* promoter and also for an unrelated SV40 minimal promoter (J.K. Polansky *et al.*, unpublished work and [39]). However, transcriptional activity was dependent on the methylation status as an *in vitro*-methylated TSDR could not enhance promoter-driven luciferase activity [54]. These data are in accordance with the findings of a similar study by Kim and Leonard [55], who additionally identified that the transcription factor cAMP-response-element-binding protein/activating transcription factor (CREB/ATF) binds in a demethylation-restricted manner to a conserved element within the TSDR.

Besides TSDR, other regions in the *Foxp3* locus could also contribute to the epigenetic control of Foxp3 expression (Figure 2.2). For example, several CpG motifs are also present in the promoter region. However, while $Foxp3^+$ Tregs display complete demethylation on all motifs at that site, $Foxp3^-CD25^-CD4^+$ T cells also show a high degree of demethylation [55], indicating that the promoter region is already in a more open chromatin state even in $Foxp3^-$ T cells, and initiation of transcription might rather be regulated by the availability or activity of the required transcription factors than by the accessibility of the promoter region. However, since the molecular mechanisms controlling Foxp3 expression are far from being understood, we still have to be aware of possible contributions to epigenetic regulation by as-yet unidentified elements, especially as epigenetic modifications at the histone level have been looked at only scarcely.

On average, the *Foxp3* locus displays a high degree of conservation between mice and humans. Thus, we tested whether we could confirm the differential methylation pattern in the human system. When we analysed $CD25^{high}CD45RA^-CD4^+$ Tregs and $CD25^-CD45RA^+CD4^+$ conventional naïve T cells we indeed approved the selectively demethylated state of the TSDR homologue in Tregs [56]. Notably, human activated naïve T cells, which transiently express FOXP3 [29–32], did not display any demethylation, allowing the discrimination between $FOXP3^+$ Tregs and recently activated effector T cells transiently expressing FOXP3 and suggesting determination of the methylation status at the TSDR as a marker to unequivocally identify 'true' Treg. As with the murine homologue, the human *FOXP3* promoter region contains several CpG motifs, which, on average, are only weakly methylated and therefore differ only slightly from the fully demethylated $FOXP3^+$ Tregs [57]. Taken together, the finding that evolutionarily conserved sequences within the *Foxp3* locus are completely and selectively demethylated upon differentiation into persistent Tregs suggests an important role for epigenetic fixation of this phenotype.

2.6 Induced Tregs: stable suppressors or transient immuno-modulators?

Although the vast majority of Tregs are probably generated in the thymus, conversion of mature $CD25^-CD4^+$ non-regulatory T cells to $Foxp3^+$ Tregs in the periphery has

also been shown to occur under various tolerogenic conditions [58]. Supposedly, the best-known phenomenon here is peripheral Treg induction after oral feeding of a foreign antigen [59–62], a mechanism which most likely contributes to immunological tolerance towards food-borne antigens. In addition, certain pathogens seem to elicit induction or expansion of suppressive T cell populations to evade protective immunity [10]. From a clinical point of view, these peripheral conversion mechanisms present a promising target for new therapeutical approaches in autoimmune settings, where more (antigen-specific) Tregs are desirable to suppress undesirable immune reactions. However, so far only little is known about the required signals for Foxp3 induction as well as on the stability and activity of these *in vivo*-induced Treg populations.

A less complex and therefore more-controllable conversion system is available *in vitro* when murine Foxp3$^-$CD25$^-$CD4$^+$ T cells are activated via their TCR in the presence of TGF-β (iTregs [63,64]). Cells from these cultures switch on Foxp3 expression and acquire a suppressive capacity after only a few days. This finding raised hope that the TGF-β-driven *in vitro* conversion system could be used to generate large numbers of Tregs from *ex vivo*-isolated peripheral naïve T cells from patients, and could be transferred back as Tregs to dampen unwanted immunity. However, as we have described recently, TGF-β-induced Foxp3 expression is unstable and strictly dependent on the inducing cytokine as most of the iTregs lost Foxp3 expression as well as suppressive activity after re-stimulation in TGF-β-free medium [39]. When we analysed the methylation status of TSDR, indeed, those instable iTregs were almost completely methylated. These data confirmed two former findings, namely that initiation of Foxp3 expression can also occur when TSDR is methylated, and that a demethylated state is required for stability of Foxp3 expression. This became even more clear in follow-up experiments, where we could stabilize TGF-β-induced Foxp3 expression by treatment with azacytidine, a nucleoside analogue, which interferes with the function of DNA methyltransferase 1 and thereby leads to rapid passive DNA demethylation [54]. Moreover, treatment of Foxp3$^-$CD25$^-$CD4$^+$ T cells with azacytidine alone was sufficient to induce Foxp3 expression, which was accompanied by efficient TSDR demethylation. Azacytidine-treated cells, which did not upregulate Foxp3 expression, also did not display changes in the methylation pattern of TSDR. This somewhat surprising result, which was also reported by others [55], suggests that an open TSDR might feed back to the promoter and thereby initiate *Foxp3* transcription, as TSDR alone does not show promoter activity in luciferase assays (J.K. Polansky *et al.*, unpublished work). Effects on other regulating elements, such as the TGF-β sensor [51], could also be imagined. Anyway, although azacytidine nicely promotes stability of Foxp3 expression, its use for therapeutical applications is hazardous since it acts globally on the whole genome in a stochastic fashion and the expression of other genes are likely to get changed unnoticed. For these reasons it is of great interest to define the signals and factors capable of inducing demethylation specifically at TSDR, leading to a stable suppressive phenotype without changing the overall pattern of gene expression. These signals obviously seem to be present in the thymus, but under the right conditions can also be generated in the periphery. We obtained evidence for the latter possibility by analysing *in vivo*-converted Tregs,

which have been induced by targeting the relevant antigen to DEC205$^+$ dendritic cells, a mode of induction previously established by Kretschmer *et al.* [65,66]. The converted cells displayed a high degree of TSDR demethylation [54] and were stable after re-stimulation *in vitro* and *in vivo* [54,65]. Whether other *in vivo*-induced Treg populations (e.g. after oral feeding) also render a stable phenotype remains to be determined. It is even conceivable that in certain settings only transient suppressors are desirable, for example to resolve an ongoing immune response after a pathogen has been cleared, but no permanent tolerance to the eliciting antigen should be established. The nature of the determining signal(s) (e.g. cytokine environment, ligand–receptor binding, duration of dendritic cell–T cell interaction) is so far completely unclear, as are the intracellular players, which induce or inhibit the remodelling of the *Foxp3* locus and subsequently the epigenetic imprinting by demethylation of TSDR. The DEC205-conversion system described above and recent reports that CD103$^+$ dendritic cells are capable of inducing Tregs in the periphery [67,68] indicate that certain dendritic cell subpopulations might by specially equipped for the induction and maybe even stabilization of Foxp3 expression in naïve T cells. In the thymus, this property might by exerted by medullary thymic epithelial cells (mTECs), which are believed to mediate Treg generation or selection [69]. One soluble factor that has recently gained attention in the Treg field is the vitamin A metabolite all-*trans*-retinoic acid, which is able to enhance TGF-β-mediated Foxp3 induction *in vitro* [67,68,70–72]. The influence of retinoic acid on the methylation status of TSDR has not been analysed yet; however, treatment with retinoic acid of human CD25$^-$CD4$^+$ naïve T cells derived from umbilical-cord blood induced histone acetylation at the *FOXP3* promoter [70]. This finding is in line with a report claiming enhanced Foxp3 stability of *in vitro*-induced retinoic acid-treated Tregs after adoptive transfer and *in vivo* re-stimulation [71]. These data strengthen the importance of histone modifications for the epigenetic control of Foxp3 expression, an idea that has been promoted by a recent publication [73]. The authors reported that *in vivo* administration of histone deacetylase-inhibitors (leading to the accumulation of acetylated histones, which usually correlate with an open chromatin structure) increased the numbers and suppressive activity of Foxp3$^+$ Tregs. In line with these data, binding of NFAT and Smad3 to the TGF-β-sensitive enhancer region in intron 1 led to the accumulation of acetylated histone 4 at the *Foxp3* promoter region [51]. Thus, although the mechanisms leading to remodelling of the *Foxp3* locus are still ill-defined, it seems that without epigenetic imprinting, manifested by the demethylated state of the evolutionarily conserved element TSDR, Foxp3 expression and thereby the suppressive capacity of Tregs remains transient.

2.7 Conclusions

Tregs are capable of suppressing immune responses of various kinds. This makes them a very promising target population for cell-therapeutic approaches mainly in autoimmune diseases and solid-organ transplantation. However, since Tregs can only be

isolated from peripheral blood in limited numbers, *in vivo* or *in vitro* conversion of naïve $CD25^{-}CD4^{+}$ T cells into Tregs seems an auspicious aim. For this conversion, the transcription factor Foxp3 plays a crucial role since it has been shown to be sufficient to confer suppressive capacity to non-regulatory T cells. Controlling Foxp3 expression therefore equals control of suppressive activity, which led to an explosive interest in the mechanisms regulating Foxp3 expression. Meanwhile, several *in vivo* and *in vitro* conversion protocols have been established, some of which yield high rates of Foxp3 induction. However, what has also been noted is that without chromatin remodelling on the *Foxp3* locus expression does not get epigenetically fixed and might be lost when induced $Foxp3^{+}$ cells encounter an adverse environment. This is of special importance when *in vitro*-induced Tregs are to be exploited in therapeutic settings and might revert to their former $Foxp3^{-}$ phenotype upon transfer into patients. Hence there is a potential epigenetic control mechanism for the *Foxp3* locus. Indeed, to date, although the molecular regulation of Foxp3 expression is still enigmatic, there are various lines of evidence highlighting a contribution of DNA (de)methylation and histone modifications to the regulation of Foxp3 expression. In particular, the selective demethylated state of an evolutionarily conserved element in the *Foxp3* locus in Tregs, that we have termed TSDR, correlates with the stability of Foxp3 expression. This site has also been found to be associated with modified histones, indicative of an open chromatin structure. Similar modifications could also be induced at the promoter region, which at the level of DNA methylation displays only marginal differences between Tregs and $Foxp3^{-}$ cells. The interplay and cross-dependency between histone modifications and DNA methylation are still poorly understood and might differ depending on the gene locus. For the *Foxp3* gene, the more dynamic mechanisms of histone modifications might rather be involved in the initiation of *Foxp3* transcription by providing accessibility of the *Foxp3* promoter for transcription factors, whereas DNA (de)methylation at TSDR might finalize the terminal differentiation into stable Foxp3-expressing Tregs. The finding that epigenetic modifications of the *Foxp3* locus critically contribute to the generation of permanently fixed $Foxp3^{+}$ Tregs might not only be exploited for the unequivocal identification of 'true' Tregs via determination of the TSDR methylation status, but might also lead to the development of novel protocols for generation *de novo* of Tregs for clinical applications in patients with autoimmune disease or allo-graft recipients.

References

1. Nishizuka, Y. and Sakakura, T. (1969) Thymus and reproduction: sex-linked dysgenesia of the gonad after neonatal thymectomy in mice. *Science* **166**, 753–755.
2. Gershon, R.K. and Kondo, K. (1970) Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology* **18**, 723–737.
3. Moller, G. (1988) Do suppressor T cells exist? *Scandinavian Journal of Immunology* **27**, 247–250.
4. Sakaguchi, S., Sakaguchi, N., Asano, M. *et al.* (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *Journal of Immunology* **155**, 1151–1164.

5. Thornton, A.M. and Shevach, E.M. (1998) $CD4^+CD25^+$ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *Journal of Experimental Medicine* **188**, 287–296.
6. Shevach, E.M. (2006) From vanilla to 28 flavors: multiple varieties of T regulatory cells. *Immunity* **25**, 195–201.
7. Roncarolo, M.G. and Battaglia, M. (2007) Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. *Nature Reviews Immunology* **7**, 585–598.
8. Yong, Z., Chang, L., Mei, Y.X. *et al.* (2007) Role and mechanisms of $CD4^+CD25^+$ regulatory T cells in the induction and maintenance of transplantation tolerance. *Transplant Immunology* **17**, 120–129.
9. Akdis, M. and Akdis, C.A. (2007) Mechanisms of allergen-specific immunotherapy. *Journal of Allergy and Clinical Immunology* **119**, 780–791.
10. Belkaid, Y. (2007) Regulatory T cells and infection: a dangerous necessity. *Nature Reviews Immunology* **7**, 875–888.
11. Curiel, T.J. (2007) Tregs and rethinking cancer immunotherapy. *Journal of Clinical Investigation* **117**, 1167–1174.
12. Tang, Q. and Bluestone, J.A. (2008) The $Foxp3^+$ regulatory T cell: a jack of all trades, master of regulation. *Nature Immunology* **9**, 239–244.
13. Pandiyan, P., Zheng, L., Ishihara, S. *et al.* (2007) $CD4^+CD25^+Foxp3^+$ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. *Nature Immunology* **8**, 1353–1362.
14. Takahashi, T., Kuniyasu, Y., Toda, M. *et al.* (1998) Immunologic self-tolerance maintained by $CD25^+CD4^+$ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *International Immunology* **10**, 1969–1980.
15. Bopp, T., Becker, C., Klein, M. *et al.* (2007) Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *Journal of Experimental Medicine* **204**, 1303–1310.
16. Cederbom, L., Hall, H. and Ivars, F. (2000) $CD4^+CD25^+$ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *European Journal of Immunology* **30**, 1538–1543.
17. Takahashi, T., Tagami, T., Yamazaki, S. *et al.* (2000) Immunologic self-tolerance maintained by $CD25^+CD4^+$ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *Journal of Experimental Medicine* **192**, 303–310.
18. Grossman, W.J., Verbsky, J.W., Barchet, W. *et al.* (2004) Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* **21**, 589–601.
19. Qin, H.Y., Mukherjee, R., Lee-Chan, E. *et al.* (2006) A novel mechanism of regulatory T cell-mediated down-regulation of autoimmunity. *International Immunology* **18**, 1001–1015.
20. Shimizu, J., Yamazaki, S., Takahashi, T. *et al.* (2002) Stimulation of $CD25^+CD4^+$ regulatory T cells through GITR breaks immunological self-tolerance. *Nature Immunology* **3**, 135–142.
21. McHugh, R., Whitters, M.J., Piccorillo, C.A. *et al.* (2002) $CD4^+CD25^+$ immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* **16**, 311–323.
22. Fontenot, J.D., Gavin, M.A. and Rudensky, A.Y. (2003) Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. *Nature Immunology* **4**, 330–336.
23. Khattri, R., Cox, T., Yasayko, S.A. *et al.* (2003) An essential role for Scurfin in $CD4^+CD25^+$ T regulatory cells. *Nature Immunology* **4**, 337–342.
24. Hori, S., Nomura, T. and Sakaguchi, S. (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057–1061.
25. Lahl, K., Loddenkemper, C., Drouin, C. *et al.* (2007) Selective depletion of $Foxp3^+$ regulatory T cells induces a scurfy-like disease. *Journal of Experimental Medicine* **204**, 57–63.

26. Wan, Y.Y. and Flavell, R.A. (2005) Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. *Proceedings of the National Academy of Sciences USA* **102**, 5126–5131.
27. Bettelli, E., Carrier, Y., Gao, W. *et al.* (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* **441**, 235–238.
28. Ziegler, S.F. (2007) FOXP3: Not just for regulatory T cells anymore. *European Journal of Immunology* **37**, 21–23.
29. Walker, M.R., Kasprowicz, D.J., Gersuk, V.H. *et al.* (2003) Induction of FoxP3 and acquisition of T regulatory activity by stimulated human $CD4^+CD25^-$ T cells. *Journal of Clinical Investigation* **112**, 1437–1443.
30. Pillai, V., Ortega, S.B., Wang, C.K. *et al.* (2007) Transient regulatory T-cells: A state attained by all activated human T-cells. *Clinical Immunology* **123**, 18–29.
31. Gavin, M.A., Torgerson, T.R., Houston, E. *et al.* (2006) Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proceedings of the National Academy of Sciences USA* **103**, 6659–6664.
32. Tran, D.Q., Ramsey, H. and Shevach, E.M. (2007) Induction of FOXP3 expression in naive human $CD4^+FOXP3^-$ T cells by T cell receptor stimulation is TGF-beta-dependent but does not confer a regulatory phenotype. *Blood* **110**, 2983–2990.
33. Brunkow, M.E., Jeffery, E.W., Hjerrild, K.A. *et al.* (2001) Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature Genetics* **27**, 68–73.
34. Ziegler, S.F. (2006) FOXP3: of mice and men. *Annual Review of Immunology* **24**, 209–226.
35. Kim, J.M., Rasmussen, J.P. and Rudensky, A.Y. (2007) Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nature Immunology* **8**, 191–197.
36. Lin, W., Haribhai, D., Relland, L.M. *et al.* (2007) Regulatory T cell development in the absence of functional Foxp3. *Nature Immunology* **8**, 359–368.
37. Gavin, M.A., Rasmussen, J.P., Fontenot, J.D. *et al.* (2007) Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* **445**, 771–775.
38. Williams, L.M. and Rudensky, A.Y. (2007) Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nature Immunology* **8**, 277–284.
39. Floess, S., Freyer, J., Siewert, C. *et al.* (2007) Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biology* **5**, e38.
40. Schubert, L.A., Jeffery, E., Zhang, Y. *et al.* (2001) Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. *Journal of Biological Chemistry* **276**, 37672–37679.
41. Marson, A., Kretschmer, K., Frampton, G.M. *et al.* (2007) Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature* **445**, 931–935.
42. Zheng, Y., Josefowicz, S.Z., Kas, A. *et al.* (2007) Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature* **445**, 936–940.
43. Wu, Y., Borde, M., Heissmeyer, V. *et al.* (2006) FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* **126**, 375–387.
44. Chen, C., Rowell, E.A., Thomas, R.M. *et al.* (2006) Transcriptional regulation by Foxp3 is associated with direct promoter occupancy and modulation of histone acetylation. *Journal of Biological Chemistry* **281**, 36828–36834.
45. Bettelli, E., Dastrange, M. and Oukka, M. (2005) Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells. *Proceedings of the National Academy of Sciences USA* **102**, 5138–5143.
46. Mantel, P.Y., Ouaked, N., Ruckert, B. *et al.* (2006) Molecular mechanisms underlying FOXP3 induction in human T cells. *Journal of Immunology* **176**, 3593–3602.

47. Mantel, P.Y., Kuipers, H., Boyman, O. *et al.* (2007) GATA3-driven Th2 responses inhibit TGF-beta1-induced FOXP3 expression and the formation of regulatory T cells. *PLoS Biology* **5**, e329.
48. Wei, J., Duramad, O., Perng, O.A. *et al.* (2007) Antagonistic nature of T helper 1/2 developmental programs in opposing peripheral induction of Foxp3$^+$ regulatory T cells. *Proceedings of the National Academy of Sciences USA* **104**, 18169–18174.
49. Zorn, E., Nelson, E.A., Mohseni, M. *et al.* (2006) IL-2 regulates FOXP3 expression in human CD4$^+$CD25$^+$ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells *in vivo*. *Blood* **108**, 1571–1579.
50. Burchill, M.A., Yang, J., Vogtenhuber, C. *et al.* (2007) IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3$^+$ regulatory T cells. *Journal of Immunology* **178**, 280–290.
51. Tone, Y., Furuuchi, K., Kojima, Y. *et al.* (2008) Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nature Immunology* **9**, 194–202.
52. Suffia, I.J., Reckling, S.K., Piccirillo, C.A. *et al.* (2006) Infected site-restricted Foxp3$^+$ natural regulatory T cells are specific for microbial antigens. *Journal of Experimental Medicine* **203**, 777–788.
53. Lee, G.R., Kim, S.T., Spilianakis, C.G. *et al.* (2006) T helper cell differentiation: regulation by cis elements and epigenetics. *Immunity* **24**, 369–379.
54. Polansky, J., Kretschmer, K., Freyer, J. *et al.* (2008) DNA methylation controls foxp3 gene expression. *European Journal of Immunology* **38**(6), 1654.
55. Kim, H.P. and Leonard, W.J. (2007) CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. *Journal of Experimental Medicine* **204**, 1543–1551.
56. Baron, U., Floess, S., Wieczorek, G. *et al.* (2007) DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3$^+$ conventional T cells. *European Journal of Immunology* **37**, 2378–2389.
57. Janson, P.C., Winerdal, M.E., Marits, P. *et al.* (2008) FOXP3 promoter demethylation reveals the committed Treg population in humans. *PLoS ONE* **3**, e1612.
58. Jaeckel, E., Kretschmer, K., Apostolou, I. *et al.* (2006) Instruction of Treg commitment in peripheral T cells is suited to reverse autoimmunity. *Seminars in Immunology* **18**, 89–92.
59. Hauet-Broere, F., Unger, W.W., Garssen, J. *et al.* (2003) Functional CD25$^-$ and CD25$^+$ mucosal regulatory T cells are induced in gut-draining lymphoid tissue within 48 h after oral antigen application. *European Journal of Immunology* **33**, 2801–2810.
60. Hultkrantz, S., Ostman, S. and Telemo, E. (2005) Induction of antigen-specific regulatory T cells in the liver-draining celiac lymph node following oral antigen administration. *Immunology* **116**, 362–372.
61. Thorstenson, K.M. and Khoruts, A. (2001) Generation of anergic and potentially immunoregulatory CD25$^+$CD4$^+$ T cells *in vivo* after induction of peripheral tolerance with intravenous or oral antigen. *Journal of Immunology* **167**, 188–195.
62. Siewert, C., Lauer, U., Cording, S. *et al.* (2008) Experience-driven development: effector/memory-like alphaE$^+$Foxp3$^+$ regulatory T cells originate from both naive T cells and naturally occurring naive-like regulatory T cells. *Journal of Immunology* **180**, 146–155.
63. Chen, W., Jin, W., Hardegen, N. *et al.* (2003) Conversion of peripheral CD4$^+$CD25$^-$ naive T cells to CD4$^+$CD25$^+$ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *Journal of Experimental Medicine* **198**, 1875–1886.
64. Fantini, M.C., Becker, C., Monteleone, G. *et al.* (2004) Cutting edge: TGF-beta induces a regulatory phenotype in CD4$^+$CD25$^-$ T cells through Foxp3 induction and down-regulation of Smad7. *Journal of Immunology* **172**, 5149–5153.

65. Kretschmer, K., Apostolou, I., Hawiger, D. *et al.* (2005) Inducing and expanding regulatory T cell populations by foreign antigen. *Nature Immunology* **12**, 1219–1227.
66. Kretschmer, K., Heng, T.S. and von Boehmer, H. (2006) De novo production of antigen-specific suppressor cells *in vivo*. *Nature Protocols* **1**, 653–661.
67. Sun, C.M., Hall, J.A., Blank, R.B. *et al.* (2007) Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 Treg cells via retinoic acid. *Journal of Experimental Medicine* **204**, 1775–1785.
68. Coombes, J.L., Siddiqui, K.R., Arancibia-Carcamo, C.V. *et al.* (2007) A functionally specialized population of mucosal CD103$^+$ DCs induces Foxp3$^+$ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *Journal of Experimental Medicine* **204**, 1757–1764.
69. Aschenbrenner, K., D'Cruz, L.M., Vollmann, E.H. *et al.* (2007) Selection of Foxp3$^+$ regulatory T cells specific for self antigen expressed and presented by Aire$^+$ medullary thymic epithelial cells. *Nature Immunology* **8**, 351–358.
70. Kang, S.G., Lim, H.W., Andrisani, O.M. *et al.* (2007) Vitamin A metabolites induce gut-homing FoxP3$^+$ regulatory T cells. *Journal of Immunology* **179**, 3724–3733.
71. Benson, M.J., Pino-Lagos, K., Rosemblatt, M. *et al.* (2007) All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *Journal of Experimental Medicine* **204**, 1765–1774.
72. Mucida, D., Park, Y., Kim, G. *et al.* (2007) Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science* **317**, 256–260.
73. Tao, R., de Zoeten, E.F., Ozkaynak, E. *et al.* (2007) Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nature Medicine* **13**, 1299–1307.

Abstract

Regulatory T cells (Tregs) harbour great therapeutic potential for the treatment of autoimmune diseases due to their potent immunosuppressive capacity. The majority of these cells express the transcription factor Foxp3, which is critical for both lineage identity and function of Tregs. We here discuss our current understanding of the molecular regulation of Foxp3 expression and the contribution of epigenetic mechanisms to the maintenance of stable Treg populations. Knowledge of these processes is of significant importance if Tregs are devised for clinical applications.

Key words: Foxp3; regulatory T cells; epigenetic gene regulation; DNA methylation; lineage stability; lineage differentiation

3

The role of NF-κB in central tolerance

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3.1 Introduction

Nuclear factor κB (NF-κB) is a family of structurally related transcription factors. In mammals, the NF-κB family consists of five members: NF-κB1 (p105/50), NF-κB2 (p100/52), RelA (p65), RelB and c-Rel. They all have a structurally conserved N-terminal 300-amino-acid Rel homology domain (RHD), which contains sequences responsible for dimerization, nuclear translocation and DNA binding. Only RelA, RelB and c-Rel have a transactivation domain, which is non-homologous and located at the C-termini of the proteins. The other two NF-κB proteins, NF-κB1 and NF-κB2, lack a transactivation domain but instead contain seven ankyrin repeats, a 33-amino-acid characteristic motif of inhibitor of NF-κB (IκB) that mediates protein–protein interactions. Although RelA, RelB and c-Rel are expressed as their mature forms, NF-κB1 and NF-κB2 are generated as precursor proteins, p105 and p100 respectively. The ankyrin repeats containing NF-κB1 (p105) and NF-κB2 (p100) can function as IκB-like proteins to retain NF-κB in the cytoplasm. The ankyrin domain is proteolytically cleaved and degraded for generation of the mature forms of NF-κB1 (p50) and NF-κB 2 (p52). The five NF-κB proteins can form 15 transcription factors through homo- or heterodimerization [1]. However, not all of them have transactivation activity. Homodimers of p50 and p52 can bind to DNA, but function as transcription repressors since they lack a transactivation domain. However, they can stimulate transcription when binding to Bcl-3, an IκB-like protein. The main activated forms of NF-κB include RelA:p50 and RelB:p52. NF-κB family members play critical roles in a

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wide variety of biological processes, including immune and inflammatory responses, development, cell growth and apoptosis.

3.2 Canonical and alternative NF-κB pathways

Two NF-κB pathways

In resting cells, NF-κB dimers are retained as inactive forms in the cytoplasm by IκB proteins, which are specific inhibitors that bind RHD through the ankyrin domain and mask the nuclear localization signal function of NF-κB. NF-κB dimers can be activated by either degradation of IκB in the canonical pathway or processing of p100 in the alternative pathway. RelA:p50 and c-Rel:p50 are the major NF-κB dimers activated by the canonical pathway. Since RelB : p50 is typically sequestrated by p100 its activation requires processing of p100, a protein that can heteromerize and sequester RelB, RelA and c-Rel [2]. Processing of p100 to p52 produces and activates the RelB:p52 heterodimer. Although RelA:p52 and c-Rel:p52 heterodimers are also produced, they are still under the control of multiple IκB inhibitors (mainly IκBα and IκBβ), whose degradation requires activation of IκB kinase (IKK) as in canonical pathway. Thus, these NF-κB dimers are at the crossroad of the canonical and alternative NF-κB pathways, which has been referred to as a hybrid pathway [3].

Activation and regulation of the canonical NF-κB pathway

The canonical pathway can be rapidly and transiently activated by a variety of stimuli. Pro-inflammatory stimuli, such as pathogen-derived lipopolysaccharide and cytokines like tumour necrosis factor α (TNF-α) and interleukin (IL)-1, are strong inducers of the canonical NF-κB pathway. Upon stimulation, IKK is activated and phosphorylates specific serines in the IκB proteins, leading to their ubiquitination and degradation, thus allowing the liberation and nuclear translocation of NF-κB dimers to induce gene expression. The primary NF-κB dimer activated by this pathway is RelA:p50, which leads to coordinate expression of multiple inflammatory and innate immune genes.

Degradation of IκBs is the characteristic feature of activation of the canonical NF-κB pathway. IκBs are a family of structurally related proteins, each of which contains multiple ankyrin repeats that interact with the nuclear-localizing signals of RHDs to prevent nuclear translocation of Rel subunits. Seven IκBs have been identified to date in mammals, including IκBα, IκBβ, IκBε, p105 (IκBγ), p100 (IκBδ), IκBζ and Bcl-3. Different IκBs display different preference of interaction with NF-κB dimers. For example, IκBα associates with a complex containing RelA or c-Rel, but not with dimers containing RelB or with homodimers of p50 or p52 [4]. In contrast, Bcl-3 preferentially binds to p50:p50 and p52:p52 homodimers [5,6]. IκBζ, the most recently identified IκB, preferentially binds the p50 subunit [7]. The formation of different complexes between IκBs and NF-κB dimers could in part generate the transcriptional specificity of target genes.

Degradation of IκBs is a ubiquitin–proteasome-dependent process. IKK is critical in IκB phosphorylation, which then triggers the ubiquitin–proteasome degradation pathway. The IKK complex contains two kinase subunits, IKKα and IKKβ, and one structural subunit, IKKγ (NEMO). Of the two kinase subunits, IKKβ is the most important for activation of the canonical NF-κB pathway [8], while lack of IKKα does not seem to affect the canonical NF-κB activity [9]. IKKγ is also required for canonical activation of the NF-κB pathway [10].

Activation of the canonical NF-κB pathway must be regulated and rapidly curtailed following stimulation, because an uncontrolled activation could result in deleterious or even fatal conditions, such as acute inflammation, septic shock, cell hyperplasia and cancer. The best-known mechanism is synthesis of IκBs, which enter the nucleus, bind and transport NF-κB dimers back to the cytoplasm and thereby prevent the transcription of inflammatory genes [11]. To ensure the efficiency of this feedback inhibition, two additional mechanisms are also employed: inactivation of IKK activity, which protects newly synthesized IκBs from degradation, and deacetylation of Rel proteins, which facilitates IκB binding [11,12].

Ubiquitination also plays an important role in activation of NF-κB pathways, and thus serves as another target for control of NF-κB activity. Different ubiquitination configurations might have different functions. The most frequently reported role of Lys-48-linked polyubiquitination is to render proteins susceptible to proteasomal degradation. By contrast, regulation of protein kinase activity and protein trafficking, such as endocytosis and lysosomal targeting, are mainly regulated by Lys-63-linked polyubiquitin chains or mono-ubiquitination [13]. To date, two de-ubiquitinating enzymes have been identified to have important roles in regulating the NF-κB pathway through modification of ubiquitination profile: A20 and CYLD. Whereas CYLD inhibits NF-κB activation by cleaving Lys-63-linked polyubiquitin chains from different proteins such as TNF-receptor-associated factor (TRAF)2, TRAF6 and NEMO [14], A20 has dual roles. It can inhibit RIP or TRAF6 activation by disassembling Lys-63-linked polyubiquitin chains, and assemble Lys-48-linked polyubiquitin chains on RIP for proteosomal degradation [15].

Other inhibitory molecules, such as Twist and PIAS1, can also inhibit RelA transactivation activity by physical interaction [16,17]. Interestingly, although Twist expression is inducible by NF-κB activity, PIAS1 is not. However, PIAS1 phosphorylation, which is required for its repression function, depends on pro-inflammatory stimuli activated by IKKα [18].

Activation and regulation of the alternative NF-κB pathway

The non-canonical (or alternative) pathway is usually triggered by non-inflammatory stimuli, such as lymphotoxin β (LTβ) signalling, CD40L, receptor activator of NF-κB ligand (RANKL) and B cell-activating factor belonging to the TNF family (BAFF). The alternative NF-κB pathway has been shown to play a central role in the expression of genes involved in development, maintenance and function of primary and secondary

lymphoid organs [19]. Activation of the alternative NF-κB pathway is strictly dependent on NF-κB-inducing kinase (NIK) and IKKα, and independent of IKKβ and IKKγ [20,21]. This could be explained by the fact NF-κB2 is phosphorylated most efficiently by IKKα. On the other hand, IκBα is phosphorylated much more efficiently by IKKβ than by IKKα and not at all by NIK [21]. NIK not only activates IKKα but also recruits IKKα to the p100 complex which results in the phosphorylation of N- and C-terminal serines of p100, and triggers p100 ubiquitination and subsequent processing [22]. Therefore, the protein kinase NIK also functions as an adapter molecule [11]. It is also important to note that NIK-mediated phosphorylation of p100 at serines 866 and 870 functions to alleviate the intrinsic inhibition mediated by the processing-inhibitory domain (PID domain) [3]. Since the RHD of p100 is most commonly associated with RelB, activation of this alternative pathway results in nuclear translocation of p52–RelB heterodimer.

Regulation of the alternative NF-κB pathway is focussed on p100 processing. First, p100 itself might bear a three-dimensional domain, which could sequestrate the C-terminal sequences of p100 required for recruitment of IKKa [11]. Second, NIK activity can be negatively regulated by both *cis*-acting sequences and *trans*-acting regulatory molecules [23,24]. Furthermore, NIK activity can be also controlled at the protein level. Several studies have suggested that TRAF3 can inhibit p100 processing by inducing NIK degradation via the proteasome [25,26]. In agreement with this, *Traf3*$^{-/-}$ cells shows constitutive p100 processing to p52 [27]. A recent study has also shown that NIK stability is maintained by heat-shock protein 90 (HSP90), and that functional inhibition of HSP90 efficiently disrupts its interaction with NIK, resulting in NIK degradation and subsequent blockade of p100 processing [28]. Finally, the multifunctional cytokine Tweak has been shown to induce p100 processing by an unknown mechanism, that is dependent on NIK, IKKα and TRAF2 [29].

Cross-regulation between canonical and alternative NF-κB signalling

The canonical and alternative NF-κB pathways do not exist in isolation and the close cross-talk contributes to fine-tuning of signalling processes. Activation of the canonical NF-κB pathway not only induces negative feedback by upregulating classical IκBs but also increases p100 and RelB expression. Like classical IκBs, p100 can also inhibit RelA-containing dimer nuclear translocation as a negative-feedback mechanism [30]. Likewise, it has also been shown that in T cells p100 limits RelA-mediated NF-κB activity following activation [31]. Increased RelB expression might also serve as a negative-feedback mechanism as discussed below. Inversely, lack of constitutive activity of IKKβ or RelA decreases the homeostasis level of p100 and RelB transcription [32]. As a functional consequence, diminished alternative NF-κB activation upon LTβ receptor (LTβR) stimulation is found in *Ikkb*$^{-/-}$ murine embryo fibroblasts [32]. Excess p50 resulting from the lack of RelA could also compete with p52 for RelB binding and contribute to the abrogated alternative NF-κB pathway activity.

RelA activity has also been shown to inhibit RelB function. Upon TNF-α stimulation, RelA can associate with RelB to dampen its *trans*-activation activity. This

RelA–RelB association is dependent on the phosphorylation of Ser-276 of RelA, in the absence of which TNF-α stimulation leads to a strong increase in the expression of endogenous NF-κB-responsive genes [33]. This complex might also serve as a negative feedback mechanism to dampen RelA activity. In fact, overexpression of RelB impairs TNF-α-induced RelA activity in murine embryo fibroblasts [34], and lack of RelB resulted in significantly increased and prolonged RelA activity and responsive gene expression [35]. However, the underlying mechanisms of RelB-mediated suppression could be more than just RelA–RelB complex formation, since IκBα stability is also reduced in the absence of RelB [35].

Competition for dimerization partners is another level of cross-regulation, given the large pool of dimers formed from NF-κB family members. For example, absence of NF-κB1/p50 leads to elevated levels of RelA:p52 and constitutive processing of p100 [32]. The elevated constitutive p100 processing, however, depletes the cellular pool of p100 complex available for alternative pathway stimulation, thereby attenuating the activation of p52-containing dimers upon LTβR stimulation [32]. Similarly, increased p50 levels are found in *Nfkb2*$^{-/-}$ murine embryo fibroblasts, leading to formation of RelB:p50 dimers. Since RelB:p50 is not subject to inhibition by classical IκBs, constitutive DNA-binding activity is found in the nucleus [32].

3.3 Thymic stroma and central tolerance

Central tolerance plays a critical role in shaping the repertoire of lymphocytes in early life to prevent autoimmunity. This occurs in the bone marrow for developing B cells and in the thymus for developing thymocytes. In spite of the complexity of how the immune system steers clear of harmful self-reactivity, there is a consensus that T cells, especially the CD4 subsets, play a central role in this process. In this chapter we will focus on the role of NF-κB in T cell central tolerance.

The thymic stroma fosters the growth, differentiation and positive and negative selection of the T cell receptor (TCR) repertoire. A proper thymic stroma microenvironment is necessary for the establishment of both central tolerance and immune competency. The thymic stromal compartment is composed of several distinct populations of cells, which include epithelium, fibroblasts, endothelium, macrophages and dendritic cells, each of which has a distinct role in T cell development. Among these populations, medullary thymic epithelial cells (mTECs) have been the most extensively investigated and shown to play a critical role in central tolerance. Importantly, NF-κB has been found to be critically involved in the regulation of mTEC development and function.

mTEC development, homeostasis and organization

The thymic medulla has gained consensus to be the main site for thymic central tolerance. This is underscored by the autoimmune phenotype observed in various mutant mouse strains that have impaired medullary characteristics: *Relb*$^{-/-}$ mice [36],

aly/aly mice [37]; *Ikka*$^{-/-}$ embryonic thymi grafted nude mice [38], *Traf6*$^{-/-}$ mice [39], *Nfkb2*$^{-/-}$ mice [40,41], *Ltbr*$^{-/-}$ mice [42,43] and *Nfkb2*$^{-/-}$*Bcl3*$^{-/-}$ mice [44]. Mice with these mutations have a disorganized thymic medulla of different degrees identified by lectin Ulex europaeus 1 (UEA1) staining: from barely detectable in *Relb*$^{-/-}$, *aly/aly* and *Traf6*$^{-/-}$ mice to a partial reduction and alternation of medulla in *Ltbr*$^{-/-}$ and *Nfkb2*$^{-/-}$ mice. It is of note that all of these factors have multiple effects on both non-hematopoietic and hematopoietic cells, which complicates the explanation of the autoimmune phenotype found in these mice. Nevertheless, by using thymic stroma transplantation and bone marrow chimaeras, investigators have largely established the essential role of these factors in the thymic stromal compartment. Interestingly, all of these molecules are involved in the NF-κB pathway, strongly supporting its critical role in mTEC development, homeostasis and organization.

Development, homeostasis and organization of mTECs are complex and involve generation of a common TEC progenitor [45], differentiation of a medullary and cortical lineage TEC progenitor, and proliferation, survival and maturation of TECs [46,47]. The current data suggest that the alternative NF-κB pathway could play a more important role than the canonical pathway in the regulation of this complex progress [36,37]. This is supported by the fact that mTECs defect often occurs in thymi with mutation of molecules in the alternative NF-κB pathway (e.g. RelB, NF-κB2, IKKα and NIK), but not the canonical pathway (e.g. NF-κB1; M. Zhu *et al.*, unpublished results).

How the NF-κB pathway regulates this progress remains largely unknown. NF-κB may not act at the very early stage of common TEC progenitor generation. Although the mTEC developmental defect is a common phenomenon in various mutant mice bearing mutation/deficiency of NF-κB-related molecules, cortical TEC development seems much less affected, if not at all. Instead, it may play a role during the branching from the common TEC progenitor to cortical TEC and mTEC progenitor cells, or at a later stage of mTEC proliferation, survival and maturation. It has been suggested that NF-κB activity is involved in TEC proliferation [48,49] and survival [48,50].

Recently, the tight-junction components claudin-3 and claudin-4 were found to be 'preferentially' expressed in autoimmune regulator (AIRE)$^{+}$ mTECs [51]. This unique mTEC population was crucial for the induction of T cell central tolerance towards tissue-restricted antigens (TRAs). Furthermore, the authors have found that the generation of this subset is unaffected in *aly/aly* or *Traf6*$^{-/-}$ mice at embryonic day 11.5. However, this population is profoundly diminished at embryonic day 16.5 in those mice. Significant higher RelB expression is also found in mTECs positive for claudin-3 and -4. These data might suggest that the proliferation and/or survival of this crucial mTEC lineage are dependent on RelB activation mediated by NIK and TRAF6. Whether mTEC proliferation and/or survival in the adult are also regulated by the same signalling pathway is unknown.

NF-κB appears to also participate in the differentiation/maturation of mTECs, a heterogeneous population expressing varying levels of major histocompatibility complex (MHC) class II, CD80 and a binding site for lectin UEA1. It is presumed that the increased level of these surface molecules indicates a progressive differentiation/maturation of mTECs, which is correlated with the function of mTECs [52].

An NF-κB-activating receptor, LTβR was suggested to participate in this process. Although *Ltbr*$^{-/-}$ thymi severely lack fully differentiated/mature UEA1-positive mTECs, the mTECs with immature appearance are largely retained [53]. Receptor activator of NF-κB (RANK) signalling was also shown to be essential for promoting the maturation of mTECs from CD80^{-} to CD80^{+} [54]. Considering that both LTβR and RANK signalling predominantly activate NF-κB, we hypothesize that NF-κB regulates the differentiation/maturation of mTECs.

It must be noted that the alternative and canonical NF-κB pathways could have a redundant role in mTEC development, homeostasis or organization. In agreement with this, *Nfkb1*$^{-/-}$*Nfkb2*$^{-/-}$ mice have a much more severe mTEC defect than *Nfkb2*$^{-/-}$ mice [40,55], whereas *Nfkb1*$^{-/-}$ mice show a normal thymus (M. Zhu *et al.*, unpublished results). However, this explanation could be complicated by the facts that NF-κB also plays a role in thymocyte development autonomously (see below) and that cross-talk between thymocytes and the stroma is critical for stromal compartment development. Although *Nfkb1*$^{-/-}$*Nfkb2*$^{-/-}$ bone marrow reconstitution in *Rag1*$^{-/-}$ mice rescued some of the defects found in straight double knockout mice, it is not known whether the mTEC defect is corrected [55]. Thus, it is still not clear whether the canonical and alternative NF-κB pathways have a redundant role intrinsic to mTECs.

Modulation of Aire regulates the expression of tissue-restricted antigens

One important function of mTECs is to present TRAs to developing thymocytes to induce negative selection of organ-specific autoreactive thymocytes [56]. The thymic expression of TRAs is controlled by both AIRE-dependent and -independent pathways [43,52,57]. Reduced thymic expression of Aire/TRA is a common defect found in all of the mutant mice mentioned above. This could be due to both cell-autonomous and developmental effects (reduced mTEC population).

Current data indicate that the alternative NF-κB pathway could regulate Aire/TRA expression through both cell-autonomous and cell-extrinsic mechanisms. Although the mTEC population is partially reduced in *Nfkb2*$^{-/-}$ mice, Aire/TRA expression is also reduced in *Nfkb2*$^{-/-}$ mTECs on a per-cell basis [40]. This could also result from altered function of thymocytes due to NF-κB2 deficiency. However, LTβR agonistic antibody treatment, which can activate the NF-κB2 signalling pathway, also upregulates Aire/TRA expression, and this effect can be abolished when the NF-κB2 pathway is ablated. Together, these data suggest that the NF-κB2 pathway can regulate Aire/TRA in a cell-autonomous manner.

Recently, RANK signalling from so-called lymphoid tissue inducer cells (LTi cells) has been found essential for autonomous Aire/TRA expression in mTECs [54]. Treatment of 2-deoxyguanosine-treated fetal thymus organ cultures with either anti-RANK antibodies or RANKL induces the emergence of CD80^{+}Aire^{+} mTECs from CD80^{-}Aire^{-}mTECs within 2 days. Given the short-term period and significant population of CD80^{+}Aire^{+} mTECs, it is likely that RANK signalling promotes the maturation of CD80^{-} mTECs to CD80^{+} mTECs and turns on Aire/TRA gene

expression. Since RANK can activate both canonical and alternative NF-κB pathways, it would be interesting to know which pathway or both RANK signalling employs to regulate Aire expression.

We have previously found that LTβR signalling is also sufficient to induce thymic Aire/TRA expression, and this appears to be dependent on NF-κB2 signalling [40,42,43]. What is the relationship between LTβR and RANK signalling pathways? Are they redundant or synergistic or do they exert their roles under different situation? These interesting questions remain to be investigated in future.

Although it has been clearly demonstrated that NF-κB activity is involved in cell-autonomous regulation of Aire/TRA, it is unknown how it works. Since there is no known NF-κB-binding site on the promoter region of the Aire gene, does this mean that NF-κB regulates Aire expression through other transcriptional factors [58] or could it be an epigenetic regulation? The underlying mechanisms remain to be elucidated.

mTECs and thymocyte migration

Despite some controversy, it is generally agreed that the thymic medulla is the major site for negative selection, especially for peripheral organ-specific autoreactive T cells. This is supported by the predominant expression of TRAs in the medulla and by the presence of co-stimulatory molecules and dendritic cells, which are important contributors to negative selection.

Trafficking of developing thymocytes in the thymus is a well-organized and ordered process. Chemokines produced by the thymic stroma cross-talk between the thymocytes and stromal cells play a pivotal role in this process. Thymocyte trafficking has been comprehensively reviewed elsewhere [59]. Here we will only discuss medullary migration. Positively selected double positive (DP) thymocytes are induced to differentiate into single positive (SP) thymocytes and begin relocating from the cortex to the medulla for negative selection and maturation. Chemotaxis directed by CCR7 and its ligand CCR7L has been shown to be important for cortex-to-medulla thymocyte migration [60,61]. Consistent with this, cortical DP thymocytes show increased CCR7 expression [61,62]; and CCR7 ligands (CCL21 and CCL19) are predominantly expressed in the thymic medulla area, especially mTECs [61,63]. Defective cortex-to-medulla migration has been found in mice deficient for CCR7 or CCR7 ligands [61], whereas the forced expression of CCR7 on premature thymocytes results in the relocation of DP thymocytes to the medulla [60]. Furthermore, impaired central tolerance was found in CCR7- and CCR7L-deficient mice [64].

Although the regulation of CCR7 expression on positively selected DP thymocytes remains largely unknown, our recent study indicates that the CCR7L expression on mTECs is partially dependent on the LTβR signalling pathway [65]. Actually, the LTβR–IKKα–alternative NF-κB signalling pathway has been well documented for the regulation of CCL21, CCL19 and other chemokines in secondary lymphoid organs and peripheral tissues [66–68]. We found that CCL21 and CCL19 are both significantly reduced in the thymi of $Ltbr^{-/-}$ mice as compared to wild-type thymi.

Re-analysis of their expression in isolated mTECs further demonstrates an intrinsic role of LTβR signalling in CCL21 and CCL19 thymic regulation. The reduction of CCL21 and CCL19 was further found to have functional consequences: more SP $CD8^+$ thymocytes accumulated in the cortex in the $Ltbr^{-/-}$ mice compared with wild-type mice [65]. This impaired medullary migration could, at least partially, contribute to the impaired negative selection of autoreactive thymocytes found in $Ltbr^{-/-}$ mice. It is worthwhile to note that although we found significant impairment of thymic negative selection in $Ltbr^{-/-}$ mice, Venanzi *et al.* [69] found only a slight or no impairment of thymic negative selection. This could be due to differences in the experimental models used. Whereas mice bearing a TCR transgene from ovalbumin-specific $CD8^+$ T cells (OT-I cells) were used in our study, mice carrying a transgene from ovalbumin-specific $CD4^+$ T cells (OT-II cells) were used by Venanzi *et al.*. It has been recently known that medullary migration of $CD8^+$ SP thymocytes relies more on CCR7 than $CD4^+$ SP thymocytes [70].

3.4 NF-κB and regulatory T cell development

$CD4^+Foxp3^+$ natural regulatory T cells (nTregs) arise in the thymus. Immunofluoresence staining of thymic sections shows that Foxp3 is predominantly expressed in the thymic medulla, suggesting a critical role of the medulla for $CD4^+Foxp3^+$ Treg development [71]. Consistently, mice with severe defects of thymic medulla, such as $Relb^{-/-}$, *aly/aly* and $Traf6^{-/-}$ mice, have dramatically reduced numbers of $Foxp3^+$ nTreg cells in thymi ([37,39] and M. Zhu *et al.*, unpublished results). However, the role of the thymic medulla for $Foxp3^+$ nTreg development and function is still vastly unknown. TRAs could be one of the signals provided by the medulla for nTreg development. A recent study from Aschenbrenner *et al.* has demonstrated that TRA-specific Tregs can be selected by $Aire^+$ mTECs, and that cross-presentation by thymic dendritic cells is not required [72]. However, this study does not exclude the possibility that thymic dendritic cells may also participate in nTreg development. As has been reported [73], thymic stromal lymphopoietin secreted by Hassal's corpuscles, structures derived from medullary epithelial cells, can stimulate thymic dendritic cells to promote the differentiation of thymocytes to Treg cells *in vitro*. Co-stimulatory and adhesion molecules expressed on thymic stromal cells are other sets of signals influencing nTreg development in the thymus. For example, deficiency in CD28, CD40, B7 or LFA-1 results in a substantial reduction in Treg cell numbers in the thymus [74]. However, given that the $Foxp3^+$ thymocytes become detectable in the late DP stage, it is not clear where and how the thymic stroma delivers and transduces signals for nTreg development. The underlying mechanism for NIK or RelB to regulate nTreg development also remains to be determined.

Although the thymic medulla could serve as a common niche for facilitation of differentiated nTregs and further functional maturation, the site for induction of nTreg differentiation could be dependent on the antigen specificity of Tregs: TRA-specific Tregs and ubiquitous antigen-specific Tregs. Whereas the former rely

more on thymic medulla for induction of differentiation, the latter could likely take place in the cortex. Supporting this view, nTregs can develop in the thymus with MHC class II expressed only in the thymic cortical epithelium [75]. Since the *Relb*$^{-/-}$ thymus shows about 50% reduction of nTregs (M. Zhu *et al.*, unpublished results), we are currently investigating whether the remaining nTregs enrich ubiquitous antigen-specific TCRs. If this is true, it would further support our hypothesis that nTreg induction could take place in both the cortex and medulla, depending on their antigen-specificity. This model would also allow us to study whether nTreg further education in the medulla is required for fully functional Treg maturation.

Whereas the alternative NF-κB pathway could influence nTreg development and function indirectly through the thymic medulla, the classical NF-κB pathway has been shown to affect nTreg development autonomously [76,77]. The study by Zheng *et al.* [76] demonstrated reduced nTregs in peripheral CD4$^+$ T cells in *p50*$^{-/-}$ *c-Rel*$^{-/-}$ mice, which could be influenced by both central and peripheral defects, whereas Schmidt-Supprian *et al.* [77] clearly demonstrated a central defect of nTreg development in mice with IKKβ deficiency only in CD4$^+$ T cells. Interestingly, the small number of remaining regulatory T cells in these mice contain many cells that have escaped Cre-mediated deletion of the loxP-flanked IKKβ alleles, which underscores the importance of IKKβ in the generation and/or maintenance of nTregs. The underlying mechanism remains unknown. However, it is not due to the inability of CD25 expression on T cells or reduced IL-2 production, as tested in the study [76]. Since nTreg cells are thought to be positively selected by agonist self-antigens in the thymus, which usually involves a strong activation of classical NF-κB (as discussed in more detail below), IKKβ could participate in TCR activation to regulate nTreg differentiation and/or maintenance. However, it is not known whether IKKβ is required quantitatively or qualitatively.

3.5 NF-κB and thymocyte positive and negative selection

Thymocytes at the DP stage undergo positive and negative selection where the affinity of the TCR for self-peptide–MHC complex is the crucial parameter that determines the fate of DP thymocytes. DP thymocytes with no or very low affinity to self will die by neglect. Those with low affinity will survive and proliferate, a process called positive selection. DP thymocytes with high affinity undergo negative selection or differentiation into regulatory T cells. In some cases, thymocytes are able to rescue failed positive selection by receptor editing (rearrangement of other TCR gene loci to produce a new TCR). Although negative selection could take place at the DP stage in the thymic cortex, as discussed above the thymic medulla is the most important selection site, where thymocytes are at the SP stage. NF-κB may play a role in both positive and negative selection.

NF-κB is normally sequestered in the cytoplasm by binding to inhibitory proteins (IκB), and TCR stimulation can lead to degradation of IκB and translocation of NF-κB to the nucleus, where it exerts its transcriptional function. Overexpression of a constitutively active super-inhibitory form of IκBα could abolish nuclear translocation of RelA, RelB and c-Rel in response to both TCR engagement and treatment with TNF-α [78]. Transgenic mice expressing a mutant form IκBα show disrupted positive selection, but normal negative selection [78]. Interestingly, it seems that only $CD8^+$ SP positive selection is affected. Another study using transgenic mice overexpressing a different mutant form of IκBα also confirmed this selective requirement of NF-κB on $CD8^+$ SP positive selection, and found that NF-κB activity is higher in $CD8^+$ SP thymocytes than in $CD4^+$ SP thymocytes [79]. The underlying mechanisms of NF-κB activity in thymocyte positive selection could reside at several levels. First, it is possible that NF-κB rescues DP thymocytes from apoptosis [80]. Second, NF-κB might help seting the TCR signalling strength threshold, and loss of NF-κB decreases the positively selecting signal to the point at which it might be insufficient for cell survival. Indeed, NF-κB inhibition by IκBα super-repressor decreases phosphorylation of ZAP70, a crucial tyrosine kinase essential for positive and negative selection of thymocytes [80]. Third, it is also possible that NF-κB regulates the expression of genes required for the maturation (differentiation) of DP thymocytes before or during positive selection [81]. Additional reports suggest a role for RelB and IKKα in the protection of DP and SP thymocytes from TCR-induced apoptosis [82,83].

Although NF-κB seems to play an anti-apoptotic role in thymocyte positive selection, it is suggested to be pro-apoptotic during negative selection. In H-Y-specific TCR transgenic male mice, DP thymocytes are largely absent as a result of negative selection. This is not due to impaired positive selection since this population is intact in female H-Y TCR transgenic mice, in which no negative signalling to self-antigens is present. However, if NF-κB is partially inhibited by an IκB super-repressor, the DP thymocytes in the male mice are partially rescued [80]. This strongly supports a pro-apoptotic role for NF-κB in negative selection.

However, another study indicated that NF-κB could have an opposite role, and that an inhibition of NF-κB is required for negative selection [84]. The authors found that a novel IκB-like NF-κB inhibitor, IκBNS, is upregulated by peptides that trigger negative selection but not by those inducing positive selection (i.e. survival) or non-selecting peptides. IκBNS blocks transcription from NF-κB reporters. Retroviral transduction of IκBNS in fetal thymic organ culture enhances TCR-triggered cell death consistent with its function in selection. A more physiological model is required to confirm the role of IκBNS in thymocyte negative selection. Even so, it has been suggested that NF-κB could have both a pro- and an anti-apoptotic role in thymocyte negative selection, which may depend on the timing and context [85].

It must be noted that most studies about NF-κB and thymocytes selection were performed using the H-Y TCR or a superantigen-specific TCR as a model sytem. Therefore, whether and how NF-κB pathways participate autonomously in the establishment of central tolerance toward TRAs remain to be determined. TRA-specific

transgenic TCR models will help to address the role for NF-κB in organ-specific central tolerance.

3.6 Conclusions and perspectives

Versatility and redundancy of the role of NF-κB to a large degree complicate the study and hamper the progress in this field of research. Not only could NF-κB have multifaceted functions in one cell, but it could also participate in a wide variety of biological processes. Some NF-κB molecules are even critical for viability (e.g. embryo or perinatal lethality of $Rela^{-/-}$, $Ikkb^{-/-}$ and $Ikka^{-/-}$ mice, and increased mortality in $Relb^{-/-}$ mice). Conditional targeting of specific NF-κB-related molecules at specific times and in specific cell populations would help to resolve these problems.

Although much has been discovered about the role of NF-κB in T cell central tolerance, how it actually works in detail remains largely unknown. For example, how is Aire expression regulated by NF-κB? Do other factors regulate Aire expression independently or together with NF-κB? Precisely how do NF-κB pathways control mTEC development?

The NF-κB family contains 15 hetero- or homodimer combinations that serve both redundant and specific functions. In addition, some IκB factors further expand the active transcriptional complex as direct nuclear mediators of NF-κB activity [86]. Given such a complex system, it is difficult to use conventional experimental approaches to address the functional differences and specificities among different NF-κB complexes. Therefore, various branches of biology such as systems biology, computational analysis, structural and molecular kinetics will be required to help address these complex questions.

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References

1. Hoffmann, A. and Baltimore, D. (2006) Circuitry of nuclear factor kappaB signaling. *Immunological Reviews* **210**, 171–186.
2. Naumann, M., Nieters, A., Hatada, E.N. *et al.* (1993) NF-kappa B precursor p100 inhibits nuclear translocation and DNA binding of NF-kappa B/rel-factors. *Oncogene* **8**, 2275–2281.
3. Dejardin, E. (2006) The alternative NF-kappaB pathway from biochemistry to biology: pitfalls and promises for future drug development. *Biochemical Pharmacology* **72**, 1161–1179.
4. Thompson, J.E., Phillips, R.J., Erdjument-Bromage, H. *et al.* (1995) I[kappa]B-[beta] regulates the persistent response in a biphasic activation of NF-[kappa]B. *Cell* **80**, 573–582.
5. Franzoso, G., Bours, V., Park, S. *et al.* (1992) The candidate oncoprotein Bcl-3 is an antagonist of pSO/NF-[kappa]B-mediated inhibition. *Nature* **359**, 339–342.

6. Bours, V., Franzoso, G., Azarenko, V. *et al.* (1993) The oncoprotein Bcl-3 directly transactivates through [kappa]B motifs via association with DNA-binding p50B homodimers. *Cell* **72**, 729–739.
7. Yamazaki, S., Muta, T. and Takeshige, K. (2001) A novel Ikappa B protein, Ikappa B-zeta, induced by proinflammatory stimuli, negatively regulates nuclear factor-kappa B in the nuclei. *Journal of Biological Chemistry* **276**, 27657–27662.
8. Li, Z.-W., Chu, W., Hu, Y. *et al.* (1999) The IKKbeta subunit of Ikappa B kinase (IKK) is essential for nuclear factor kappa B activation and prevention of apoptosis. *Journal of Experimental Medicine* **189**, 1839–1845.
9. Hu, Y., Baud, V., Delhase, M. *et al.* (1999) Abnormal morphogenesis but intact IKK activation in mice lacking the IKK subunit of IB kinase. *Science* **284**, 316–320.
10. Makris, C., Godfrey, V.L., Krahn-Senftleben, G. *et al.* (2000) Female mice heterozygous for IKK [gamma]/NEMO deficiencies develop a dermatopathy similar to the human X-linked disorder incontinentia pigmenti. *Molecular Cell* **5**, 969–979.
11. Xiao, G., Rabson, A.B., Young, W. *et al.* (2006) Alternative pathways of NF-kappaB activation: a double-edged sword in health and disease. *Cytokine & Growth Factor Reviews* **17**, 281–293.
12. Chen, L.-F. and Greene, W.C. (2004) Shaping the nuclear action of NF-[kappa]B. *Nature Reviews Molecular Cell Biology* **5**, 392–401.
13. Heyninck, K. and Beyaert, R. (2005) A20 inhibits NF-[kappa]B activation by dual ubiquitin-editing functions. *Trends in Biochemical Sciences* **30**, 1–4.
14. Brummelkamp, T.R., Nijman, S.M.B., Dirac, A.M.G. *et al.* (2003) Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-[kappa]B. *Nature* **424**, 797–801.
15. Wertz, I.E., O'Rourke, K.M., Zhou, H. *et al.* (2004) De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-[kappa]B signalling. *Nature* **430**, 694–699.
16. Sosic, D., Richardson, J.A., Yu, K. *et al.* (2003) Twist regulates cytokine gene expression through a negative feedback loop that represses NF-[kappa]B activity. *Cell* **112**, 169–180.
17. Liu, B., Yang, R., Wong, K.A. *et al.* (2005) Negative regulation of NF-{kappa}B signaling by PIAS1. *Molecular and Cellular Biology* **25**, 1113–1123.
18. Liu, B., Yang, Y., Chernishof, V. *et al.* (2007) Proinflammatory stimuli induce IKK[alpha]-mediated phosphorylation of PIAS1 to restrict inflammation and immunity. *Cell* **129**, 903–914.
19. Weih, F. and Caamano, J. (2003) Regulation of secondary lymphoid organ development by the nuclear factor-kappaB signal transduction pathway. *Immunological Reviews* **195**, 91–105.
20. Xiao, G., Harhaj, E.W. and Sun, S.C. (2001) NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. *Molecular Cell* **7**, 401–409.
21. Senftleben, U., Cao, Y., Xiao, G. *et al.* (2001) Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science* **293**, 1495–1499.
22. Xiao, G., Fong, A. and Sun, S.C. (2004) Induction of p100 processing by NF-kappaB-inducing kinase involves docking IkappaB kinase alpha (IKKalpha) to p100 and IKKalpha-mediated phosphorylation. *Journal of Biological Chemistry* **279**, 30099–30105.
23. Xiao, G. and Sun, S.C. (2000) Negative regulation of the nuclear factor kappa B-inducing kinase by a cis-acting domain. *Journal of Biological Chemistry* **275**, 21081–21085.
24. Hu, W.H., Mo, X.M., Walters, W.M. *et al.* (2004) TNAP, a novel repressor of NF-kappaB-inducing kinase, suppresses NF-kappaB activation. *Journal of Biological Chemistry* **279**, 35975–35983.
25. He, J.Q., Saha, S.K., Kang, J.R. *et al.* (2007) Specificity of TRAF3 in its negative regulation of the noncanonical NF-{kappa}B pathway. *Journal of Biological Chemistry* **282**, 3688–3694.
26. Liao, G., Zhang, M., Harhaj, E.W. *et al.* (2004) Regulation of the NF-{kappa}B-inducing kinase by tumor necrosis factor receptor-associated factor 3-induced degradation. *Journal of Biological Chemistry* **279**, 26243–26250.
27. He, J.Q., Zarnegar, B., Oganesyan, G. *et al.* (2006) Rescue of TRAF3-null mice by p100 NF-{kappa}B deficiency. *Journal of Experimental Medicine* **203**, 2413–2418.
28. Qing, G., Yan, P., Qu, Z. *et al.* (2007) Hsp90 regulates processing of NF-[kappa]B2 p100 involving protection of NF-[kappa]B-inducing kinase (NIK) from autophagy-mediated degradation. *Cell Research* **17**, 520–530.

29. Saitoh, T., Nakayama, M., Nakano, H. *et al.* (2003) TWEAK Induces NF-{kappa}B2 p100 processing and long lasting NF-{kappa}B activation. *Journal of Biological Chemistry* **278**, 36005–36012.
30. Basak, S., Kim, H., Kearns, J.D. *et al.* (2007) A fourth I[kappa]B protein within the NF-[kappa]B signaling modulE. *Cell* **128**, 369–381.
31. Ishimaru, N., Kishimoto, H., Hayashi, Y. *et al.* (2006) Regulation of naive T cell function by the NF-kappaB2 pathway. *Nature Immunology* **7**, 763–772.
32. Basak, S., Shih, V.F.-S. and Hoffmann, A. (2008) Generation and activation of multiple dimeric transcription factors within the NF-{kappa}B signaling system. *Molecular and Cellular Biology* **28**, 3139–3150.
33. Jacque, E., Tchenio, T., Piton, G. *et al.* (2005) RelA repression of RelB activity induces selective gene activation downstream of TNF receptors. *Proceedings of the National Academy of Sciences USA* **102**, 14635–14640.
34. Marienfeld, R., May, M.J., Berberich, I. *et al.* (2003) RelB forms transcriptionally inactive complexes with RelA/p65. *Journal of Biological Chemistry* **278**, 19852–19860.
35. Xia, Y., Chen, S., Wang, Y. *et al.* (1999) RelB Modulation of Ikappa Balpha stability as a mechanism of transcription suppression of interleukin-1alpha (IL-1alpha), IL-1beta, and tumor necrosis factor alpha in fibroblasts. *Molecular and Cellular Biology* **19**, 7688–7696.
36. Burkly, L., Hession, C., Ogata, L. *et al.* (1995) Expression of relB is required for the development of thymic medulla and dendritic cells. *Nature* **373**, 531–536.
37. Kajiura, F., Sun, S., Nomura, T. *et al.* (2004) NF-kappa B-inducing kinase establishes self-tolerance in a thymic stroma-dependent manner. *Journal of Immunology* **172**, 2067–2075.
38. Kinoshita, D., Hirota, F., Kaisho, T. *et al.* (2006) Essential role of IkappaB kinase alpha in thymic organogenesis required for the establishment of self-tolerance. *Journal of Immunology* **176**, 3995–4002.
39. Akiyama, T., Maeda, S., Yamane, S. *et al.* (2005) Dependence of self-tolerance on TRAF6-directed development of thymic stroma. *Science* **308**, 248–251.
40. Zhu, M., Chin, R.K., Christiansen, P.A. *et al.* (2006) NF-kappaB2 is required for the establishment of central tolerance through an Aire-dependent pathway. *Journal of Clinical Investigation* **116**, 2964–2971.
41. Zhang, B., Wang, Z., Ding, J. *et al.* (2006) NF-{kappa}B2 is required for the control of autoimmunity by regulating the development of medullary thymic epithelial cells. *Journal of Biological Chemistry* **281**, 38617–38624.
42. Chin, R.K., Lo, J.C., Kim, O. *et al.* (2003) Lymphotoxin pathway directs thymic Aire expression. *Nature Immunology* **4**, 1121–1127.
43. Chin, R.K., Zhu, M., Christiansen, P.A. *et al.* (2006) Lymphotoxin pathway-directed, autoimmune regulator-independent central tolerance to arthritogenic collagen. *Journal of Immunology* **177**, 290–297.
44. Zhang, X., Wang, H., Claudio, E. *et al.* (2007) A role for the I[kappa]B family member Bcl-3 in the control of central immunologic tolerance. *Immunity* **27**, 438–452.
45. Rodewald, H.-R. (2008) Thymus organogenesis. *Annual Review of Immunology* **26**, 355–388.
46. Gray, D., Abramson, J., Benoist, C. *et al.* (2007) Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire. *Journal of Experimental Medicine* **204**, 2521–2528.
47. Gray, D.H., Seach, N., Ueno, T. *et al.* (2006) Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells. *Blood* **108**, 3777–3785.
48. Lee, H.W., Park, H.K., Na, Y.J. *et al.* (2008) RANKL stimulates proliferation, adhesion and IL-7 expression of thymic epithelial cells. *Experimental & Molecular Medicine* **40**, 59–70.
49. Rossi, S.W., Jeker, L.T., Ueno, T. *et al.* (2007) Keratinocyte growth factor (KGF) enhances postnatal T-cell development via enhancements in proliferation and function of thymic epithelial cells. *Blood* **109**, 3803–3811.
50. Scupoli, M.T., Fiorini, E., Marchisio, P.C. *et al.* (2000) Lymphoid adhesion promotes human thymic epithelial cell survival via NF-(kappa)B activation. *Journal of Cell Science* **113**, 169–177.
51. Hamazaki, Y., Fujita, H., Kobayashi, T. *et al.* (2007) Medullary thymic epithelial cells expressing Aire represent a unique lineage derived from cells expressing claudin. *Nature Immunology* **8**, 304–311.

52. Derbinski, J., Gabler, J., Brors, B. *et al.* (2005) Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *Journal of Experimental Medicine* **202**, 33–45.
53. Milicevic, N.M., Nohroudi, K., Milicevic, Z. *et al.* (2008) Activation of cortical and inhibited differentiation of medullary epithelial cells in the thymus of lymphotoxin-beta receptor-deficient mice: an ultrastructural study. *Journal of Anatomy* **212**, 114–124.
54. Rossi, S.W., Kim, M.Y., Leibbrandt, A. *et al.* (2007) RANK signals from CD4(+)3(−) inducer cells regulate development of Aire-expressing epithelial cells in the thymic medulla. *Journal of Experimental Medicine* **204**, 1267–1272.
55. Franzoso, G., Carlson, L., Xing, L. *et al.* (1997) Requirement for NF-kappaB in osteoclast and B-cell development. *Genes & Development* **11**, 3482–3496.
56. Kyewski, B. and Klein, L. (2006) A central role for central tolerance. *Annual Review of Immunology* **24**, 571–606.
57. Anderson, M.S., Venanzi, E.S., Klein, L. *et al.* (2002) Projection of an immunological self shadow within the thymus by the aire protein. *Science* **298**, 1395–1401.
58. Murumagi, A., Vahamurto, P. and Peterson, P. (2003) Characterization of regulatory elements and methylation pattern of the autoimmune regulator (AIRE) promoter. *Journal of Biological Chemistry* **278**, 19784–19790.
59. Takahama, Y. (2006) Journey through the thymus: stromal guides for T-cell development and selection. *Nature Reviews Immunology* **6**, 127–135.
60. Kwan, J. and Killeen, N. (2004) CCR7 directs the migration of thymocytes into the thymic medulla. *Journal of Immunology* **172**, 3999–4007.
61. Ueno, T., Saito, F., Gray, D.H. *et al.* (2004) CCR7 signals are essential for cortex-medulla migration of developing thymocytes. *Journal of Experimental Medicine* **200**, 493–505.
62. Campbell, J.J., Pan, J. and Butcher, E.C. (1999) Cutting edge: developmental switches in chemokine responses during T cell maturation. *Journal of Immunology* **163**, 2353–2357.
63. Misslitz, A., Pabst, O., Hintzen, G. *et al.* (2004) Thymic T cell development and progenitor localization depend on CCR7. *Journal of Experimental Medicine* **200**, 481–491.
64. Kurobe, H., Liu, C., Ueno, T. *et al.* (2006) CCR7-dependent cortex-to-medulla migration of positively selected thymocytes is essential for establishing central tolerance. *Immunity* **24**, 165–177.
65. Zhu, M., Chin, R.K., Tumanov, A.V. *et al.* (2007) Lymphotoxin beta receptor is required for the migration and selection of autoreactive T cells in thymic medulla. *Journal of Immunology* **179**, 8069–8075.
66. Drayton, D.L., Bonizzi, G., Ying, X. *et al.* (2004) I{kappa}B kinase complex {alpha} kinase activity controls chemokine and high endothelial venule gene expression in lymph nodes and nasal-associated lymphoid tissue. *Journal of Immunology* **173**, 6161–6168.
67. Weih, D.S., Yilmaz, Z.B. and Weih, F. (2001) Essential role of RelB in germinal center and marginal zone formation and proper expression of homing chemokines. *Journal of Immunology* **167**, 1909–1919.
68. Lo, J.C., Chin, R.K., Lee, Y. *et al.* (2003) Differential regulation of CCL21 in lymphoid/nonlymphoid tissues for effectively attracting T cells to peripheral tissues. *Journal of Clinical Investigation* **112**, 1495–1505.
69. Venanzi, E.S., Gray, D.H., Benoist, C. *et al.* (2007) Lymphotoxin pathway and Aire influences on thymic medullary epithelial cells are unconnected. *Journal of Immunology* **179**, 5693–5700.
70. Davalos-Misslitz, A.C.M., Worbs, T., Willenzon, S. *et al.* (2007) Impaired responsiveness to T-cell receptor stimulation and defective negative selection of thymocytes in CCR7-deficient mice. *Blood* **110**, 4351–4359.
71. Fontenot, J.D., Rasmussen, J.P., Williams, L.M. *et al.* (2005) Regulatory T cell lineage specification by the forkhead transcription factor Foxp3. *Immunity* **22**, 329–341.
72. Aschenbrenner, K., D'Cruz, L.M., Vollmann, E.H. *et al.* (2007) Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells. *Nature Immunology* **8**, 351–358.
73. Watanabe, N., Wang, Y.-H., Lee, H.K. *et al.* (2005) Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. *Nature* **436**, 1181–1185.
74. Sakaguchi, S., Ono, M., Setoguchi, R. *et al.* (2006) Foxp3+CD25+CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunological Reviews* **212**, 8–27.

75. Bensinger, S.J., Bandeira, A., Jordan, M.S. *et al.* (2001) Major histocompatibility complex class II-positive cortical epithelium mediates the selection of CD4 + 25 + immunoregulatory T cells. *Journal of Experimental Medicine* **194**, 427–438.
76. Zheng, Y., Vig, M., Lyons, J. *et al.* (2003) Combined deficiency of p50 and cRel in CD4 + T cells reveals an essential requirement for nuclear factor {kappa}B in regulating mature T cell survival and in vivo function. *Journal of Experimental Medicine* **197**, 861–874.
77. Schmidt-Supprian, M., Courtois, G., Tian, J. *et al.* (2003) Mature T cells depend on signaling through the IKK complex. *Immunity* **19**, 377–389.
78. Hettmann, T. and Leiden, J.M. (2000) NF-{kappa}B is required for the positive selection of CD8 + thymocytes. *Journal of Immunology* **165**, 5004–5010.
79. Voll, R.E., Jimi, E., Phillips, R.J. *et al.* (2000) NF-[kappa]B activation by the pre-T cell receptor serves as a selective survival signal in T lymphocyte development. *Immunity* **13**, 677–689.
80. Mora, A.L., Stanley, S., Armistead, W. *et al.* (2001) Inefficient ZAP-70 phosphorylation and decreased thymic selection in vivo result from inhibition of NF-{kappa}B/Rel. *Journal of Immunology* **167**, 5628–5635.
81. Esslinger, C.W., Jongeneel, C.V. and MacDonald, H.R. (1998) Survival-independent function of NF-[kappa]B[-45 degree rule]Rel during late stages of thymocyte differentiation. *Molecular Immunology* **35**, 847–852.
82. Ren, H., Schmalstieg, A., van Oers, N.S.C. *et al.* (2002) I-{kappa}B Kinases {alpha} and {beta} have distinct roles in regulating murine T cell function. *Journal of Immunology* **168**, 3721–3731.
83. Guerin, S., Baron, M.L., Valero, R. *et al.* (2002) RelB reduces thymocyte apoptosis and regulates terminal thymocyte maturation. *European Journal of Immunology* **32**, 1–9.
84. Fiorini, E., Schmitz, I., Marissen, W.E. *et al.* (2002) Peptide-induced negative selection of thymocytes activates transcription of an NF-[kappa]B inhibitor. *Molecular Cell* **9**, 637–648.
85. Siebenlist, U., Brown, K. and Claudio, E. (2005) Control of lymphocyte development by nuclear factor-kappaB. *Nature Reviews Immunology* **5**, 435–445.
86. Bates, P.W. and Miyamoto, S. (2004) Expanded nuclear roles for I{kappa}Bs. *Science's STKE: Signal Transduction Knowledge Environment* **2004**, pe48.

Abstract

The nuclear factor κB (NF-κB) transcription factor family plays a critical role in regulating the immune system. Aberrant NF-κB signalling is frequently associated with inflammation and autoimmune diseases but the mechanisms are unclear. Two NF-κB signalling pathways have been identified: the canonical pathway and the alternative pathway. However, these two signalling pathways do not exist in isolation and cross-regulation commonly occurs. Both pathways play important roles in establishing central tolerance. The alternative pathway appears to be the key signalling component in stromal cells, while the canonical pathway exerts its function more in autonomous T cells. Increased signalling of the canonical pathway is often associated with increased T cell activation, and inflammation and recent studies show that lack of signalling of the alternative pathway could also lead to increased inflammation by impairing central tolerance.

Key words: autoimmunity; thymus; T cell development; stromal cells; regulatory T cells; chemokines; lymphotoxin; NF-κB; Aire

4

The role of Act1 in the control of autoimmunity

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4.1 Introduction

Autoimmune diseases can be divided into two major types: multi-organ (systemic) and organ-specific. The first category includes, among others, systemic lupus erythematosus (SLE), the prototypic systemic autoimmune disease, and Sjögren's syndrome. In both SLE and Sjögren's syndrome several organs can be involved and the main hallmarks of the diseases and B cell hyper-activation are the presence of antibodies specific to widely expressed nuclear antigens such as DNA, RNA, histones, SSA/Ro and SSB/La. Within the second category falls diseases such as multiple sclerosis, thyroiditis and type 1 diabetes, which target the brain, thyroid gland and pancreas, respectively. These diseases tend to rely on T cell-mediated damage as well as B cell-dependent antibody production.

The aetiology of autoimmune diseases remains largely unexplained and environmental factors, combined with both gender and individual genetics, have been implied to play important roles in disease development. Single-gene polymorphisms and deletions have been associated with autoimmunity in both humans and mouse models of autoimmunity. Based on genetic studies, some genes have been identified to have a uniform effect on both systemic and organ-specific autoimmunity, although such examples are relatively rare. Even rarer are genes that differentially affect systemic and organ-specific autoimmunity. One such gene is *Act1*, which is capable of inhibiting systemic autoimmunity while promoting organ-specific

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autoimmunity in mouse models. This chapter will describe the known mechanisms by which Act1 controls the development of autoimmunity.

4.2 Autoimmunity and autoimmune mouse models

Systemic lupus erythematosus

Patients with SLE are commonly females between 15 and 45 years of age. Patients may develop an array of symptoms including kidney, lung, brain and heart problems, all of which are thought to be mediated through the deposition of antibody–antigen complexes known as immune complexes. Other potential symptoms of SLE include ultraviolet-light sensitivity, which may lead to the development of discoid lupus. Human SLE development, like most of the other systemic autoimmune diseases, depends on a combination of multiple genetic and environmental factors. Accumulating evidence suggests that the environment has the potential to modulate individual gene expression through epigenetic mechanisms, as well as the interactions among genes, thus contributing to disease development.

The complex presentation of the disorder has resulted in the study of SLE being equally complicated, and thus the identification and development of mouse models of lupus-like disease has been of outmost importance for the understanding of the complexity of the disease. Mouse lupus-like disease is typically associated with hyperactive B cells and uncontrolled differentiation of plasma cells, resulting in elevated levels of circulating antibodies directed against self-antigens; that is, autoantibodies. Among the most studied models of SLE is the New Zealand hybrid mouse model (New Zealand black $\times$ New Zealand white) F_1 and its derivatives (reviewed in [1]). The disease in this model is characterized by spontaneous B cell activation, elevated levels of autoantibodies towards nuclear antigens and the development of immune complex-mediated kidney disease. Genetic studies aiming at defining the gene responsible for the disease quickly demonstrated that the disease is dependent on a combination of several disease-susceptibility genes, including genes located within the major histocompatibility complex (*MHC*) locus on chromosome 17 and on distal chromosome 1 (the *Sle1*/*Nba2* locus) [2–5]. Both of these loci are highly conserved in humans and much research has been performed to decipher the exact genetic component(s) located within these loci.

In the 1970s and 1980s another mouse model of lupus-like disease became available. This time, disease developed in a strain carrying a null mutation in the *Tnfrsf6* gene encoding Fas antigen [6,7]. The mutation was called *lpr* (*lymphoproliferative*) and mice homozygous (*lpr*/*lpr*) for the mutation spontaneously developed elevated levels of anti-nuclear antibodies and multi-organ infiltrations of immune cells. The mice succumbed to the disease due to immune complex-mediated kidney failure just like the New Zealand hybrid mice.

Sjögren's syndrome

Sjögren's syndrome is a relatively frequent (≈1.2% of the population) systemic autoimmune disease in which the salivary and lacrimal glands become infiltrated by mononuclear cells leading to the development of xerostomia and xerophthalmia (dry mouth and dry eye) [8]. The diagnosis of Sjögren's syndrome is dependent on the presence of elevated levels of specific autoantibodies against SSA/Ro and SSB/La. Although multiple factors, including environmental stress, viral infections and genetic background, have been proposed, the aetiology of the disease is still largely unknown.

Sjögren's syndrome is classified as primary or secondary depending on the absence or presence of other systemic autoimmune diseases such as SLE, rheumatoid arthritis or scleroderma. There is no good mouse model of *primary* Sjögren's syndrome, although many of the mouse models of SLE also develop a *secondary* Sjögren's syndrome-like infiltrate of the glands. This includes spontaneous models such as the New Zealand hybrids, MRL-*lpr/lpr* and the non-obese diabetic (NOD) mouse model of type 1 diabetes as well as several genetically altered (gene-targeted) mice.

Multiple sclerosis and experimental autoimmune encephalomyelitis

Multiple sclerosis is a devastating organ-specific autoimmune disease in which the blood–brain barrier is damaged, allowing for immune-cell infiltration into the brain, an otherwise immune-quiescent organ. The infiltrating cells subsequently destroy myelin sheaths of the nerve fibres, resulting in progressive paralysis [9]. There are four major types of multiple sclerosis: relapsing-remitting, where patients experience flares of disease followed by periods of recovery; primary-progressive, where patients experience a steady worsening of disease with no relapses or remissions; secondary-progressive, where patients revert from relapsing-remitting to progressive; and progressive-relapsing, where patients get steadily worse while still experiencing relapses and remissions. The most common treatment of relapsing-remitting multiple sclerosis is subcutaneous injections with interferon-β analogues, although only about 50% of patients respond well to the treatment. In responders, the interferon-β treatment leads to less frequent and less severe relapses and reduces the amount of non-repairable damage; however, the mechanism is still unknown.

Experimental autoimmune encephalomyelitis (EAE) is a broadly used model of antigen-induced central nervous system-specific infiltration of immune cells [10]. The antigens most often used to induce EAE in mice are peptides from proteolipoprotein (PLP139–151) and myelin oligodendrocyte glycoprotein (MOG35–55). Although several mouse strains are responsive to the treatment and develop a multiple sclerosis-like disease, some mice are resistant to disease induction [11], suggesting that genetic predisposition may play a role. Responsive mouse models develop many of the symptoms observed in multiple sclerosis patients, making them good models in which to study the effect of individual genes by either gene-targeting events (knockout or small interfering RNA (siRNA) treatment studies) or by

transgenic studies (where particular genes are over-expressed either ubiquitously or in a cell-type-specific manner).

4.3 Molecular mechanisms of autoimmunity

B cell signalling and systemic autoimmunity

Autoimmunity is initiated by the disruption of central or peripheral tolerance, resulting in the escape of autoreactive T and B cells from normal selection. These autoreactive T and B cells are activated and expanded when they encounter their cognate self-antigens and result in humoral and cellular abnormalities. Consequently, abnormalities in B cells (including increased cell survival and production of autoantibodies) often lead to pathogenic immune responses and potentially to the development of systemic autoimmune diseases. BAFF (B cell-activating factor belonging to the tumour necrosis factor (TNF) family) and its receptor (BAFFR), and CD40 ligand (CD40L)/CD40, members of TNF/TNF receptor (TNFR) superfamily, are required for B cell maturation and T cell-dependent B cell activation, respectively [12–15]. Dysregulation of BAFF/BAFFR or CD40L/CD40 pathways contributes to the escape of autoreactive B cells from normal selection, leading to the development and pathogenesis of systemic autoimmunity [16,17]. For example, BAFF transgenic mice displayed mature B cell hyperplasia and symptoms of SLE and Sjögren's syndrome, while CD40L transgenic mice developed B cell hyperplasia and lupus nephritis. In that regard it is interesting to note that patients with SLE and Sjögren's syndrome have been found to express elevated levels of serum BAFF [18].

CD40 and BAFFR utilize the adaptor TNFR-associated factor (TRAF) molecules to mediate the activation of the inhibitor of nuclear factor κB (IκB) kinase (IKK) complex (IKKα and IKKβ), resulting in activation of the nuclear factor κB (NF-κB) family of transcription factors, which are critical for the regulation of B cell survival and development [19–23]. IKKβ is mainly responsible for IκB phosphorylation, which leads to rapid ubiquitination and degradation of IκB and nuclear translocation of NF-κB (p65/p50). Conditional deletion of *Ikkb* results in a rapid loss of B cells, indicating that the *canonical* NF-κB activation pathway mediated by IKKβ is probably required for the differentiation and homeostasis of B cells [24,25].

CD40 and BAFFR are also able to induce a *non-canonical* NF-κB activation pathway; that is, the phosphorylation and processing of p100 by IKKα. This pathway is thought to depend on the activation of IKKα by NF-κB-inducing kinase (NIK), hereby resulting in the formation of an active RelB/p52 heterodimer [26–31]. Mature B cell numbers are reduced in irradiated mice reconstituted with IKKα-deficient lymphocytes, indicating the important role of this non-canonical NF-κB activation pathway in CD40- and BAFFR-mediated B cell survival [27,32]. Taken together, it is conceivable that dysregulation of CD40- and/or BAFFR-mediated canonical and/or non-canonical NF-κB activation can lead to abnormalities in B cells, hereby resulting in B cell-mediated systemic autoimmunity.

CD4⁺ T cells and organ-specific autoimmunity

$CD4^+$ T helper (Th) lymphocytes play essential regulatory roles in immune responses and autoimmune and inflammatory diseases. Upon activation by professional antigen-presenting cells, naïve $CD4^+$ Th cells can differentiate into three subsets depending on the local cytokine environment: Th1, characterized by production of interferon-γ which mediates cellular immunity, Th2 cells that synthesize interleukin (IL)-4, IL-5 and IL-13 and perform effector functions in humoral immunity and allergic responses, and Th17 cells that make IL-17 (Figure 4.1). IL-17 is a pro-inflammatory cytokine that upregulates the expression of inflammatory genes in tissue-resident cells (including fibroblasts, endothelial cells, macrophages, epithelial cells and astrocytes). Interestingly, IL-17 levels are elevated in patients with autoimmune diseases, including rheumatoid arthritis and inflammatory bowel disease [33,34]. The pathogenic role of IL-17 has been further demonstrated in experiments with IL-17-deficient mice, in which various autoimmune inflammatory disorders were suppressed [35,36].

Act1, a double-edged sword?

Although numerous genes have been shown to be important for EAE development in mice, only a few of these overlap with the genes identified in systemic autoimmunity. Genes with such an effect include *Traf3ip2* (Act1), *Tnfrsf6* (Fas), *Tnfsf6* (FasL), *Pdcd1*

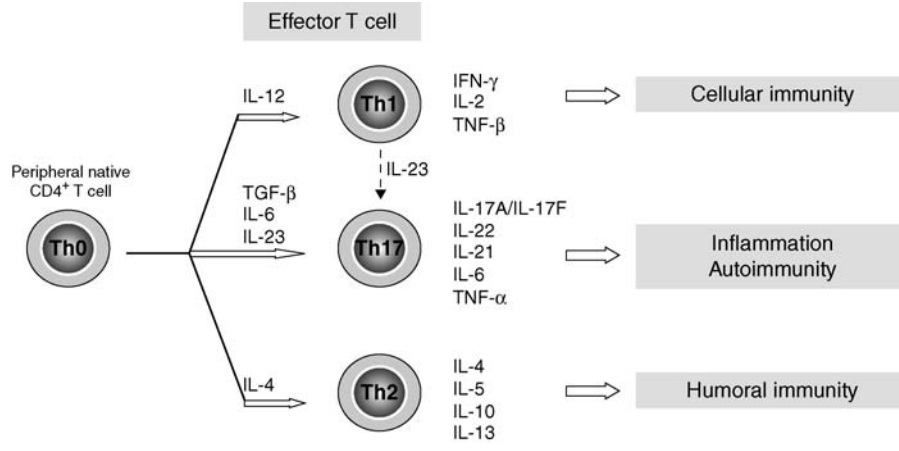

Figure 4.1 $CD4^+$ T helper cell differentiation pathways. CD4 T helper (Th) lymphocytes play essential regulatory roles in immune responses and autoimmune and inflammatory diseases. Upon activation by professional antigen-presenting cells, naïve CD4 Th cells differentiate into three lineages: Th1 (which produce interferon γ (IFN-γ), mediating cellular immunity); Th2 cells (synthesizing IL-4, IL-5 and IL-13, which leads to humoral immunity and allergic responses) and Th17 (producing IL-17, which mediates inflammatory responses). TGF-β, transforming growth factor β.

Table 4.1 Gene manipulations that trigger lupus or EAE development in healthy mice.

Gene[1]	Genetic manipulation	Expression pattern[2]	Lupus development[3]	EAE phenotype[4]	Reference
Traf3ip2 (Act1)	Knockout	Leukocytes	Yes	Resistance	[37,38]
Tnfrsf6 (Fas)	Loss of function	Leukocytes	Yes	Resistance	[39,40]
Tnfsf6 (FasL)	Loss of function	T cell	Yes	Resistance	[40,41]
Pdcd1 (PD-1)	Knockout	T cell	Yes	Resistance	[42,43]
Socs1	Knockout	T cell	Yes	Resistance	[44,45]

[1]Official gene name (synonym).
[2]Based on published data and data from Novartis Symatlas database (http://symatlas.gnf.org/SymAtlas/).
[3]Spontaneous lupus-like disease development in healthy non-autoimmune strains.
[4]Myelin oligodendrocyte glycoprotein (MOG)-induced EAE.

(PD-1) and *Socs1*, which when deleted lead to inhibited EAE as well as spontaneous lupus-like disease development [37–45] (and see Table 4.1). It should be noted that patients suffering from both multiple sclerosis and SLE are relatively rare, thus supporting the involvement of genes with opposite effects.

The remainder of this chapter will focus on the role of the adapter molecule Act1 in regulation of autoimmunity through its impact on both T and B cell-mediated immune responses. Based on a number of published studies it seems likely that Act1 controls mouse lupus and contributes to EAE development through different mechanisms reflecting the B and T cell requirements for these diseases, respectively. It was previously reported that Act1 is an important negative regulator for B cell-mediated humoral immune responses through its impact on CD40L and BAFF signalling [37]. In contrast, recent studies have shown that Act1 is a key component for the IL-17 signalling pathway and essential for Th17-mediated autoimmune and inflammatory responses [38]. The dual functions of Act1 are displayed by the Act1-deficient mice that developed B cell-mediated autoimmune phenotypes (including a dramatic increase in peripheral B cells, lymphadenopathy and splenomegaly, hypergammaglobulinaemia and Sjögren's syndrome in association with lupus nephritis), but showed resistance to Th17-dependent EAE.

4.4 Act1: a modulator of autoimmunity

Identification and structure

Act1, also known as *Traf3ip2* and *Ciks*, is a gene that has the capability to modulate both systemic and organ-specific autoimmunity. *Act1* was originally cloned as a component of the IKK/NF-κB signalling cascade [46,47]. The human *ACT1* (*TRAF3IP2*) gene encodes two isoforms of ACT1: isoform 1 (574 amino acids) and isoform 2 (565 amono acids), which lacks an alternative in-frame exon at its N-terminus. The

Figure 4.2 Structural domains of ACT1. Shown are the location of a helix–loop–helix (HLH) domain (amino acids 135–190), a coiled-coil (C-C) domain (amino acids 420–500), a SEFIR domain (amino acids 394–574) and the two identified TRAF-binding sites (EEESE, amino acids 38–42; EEERPA, amino acids 324–329).

mouse gene homologue *Act1* (*Traf3ip2*) encodes a 555-amino-acid protein product, which shares less than 80% homology with the human ACT1 (isoform 1). Studies of the Act1 structure have revealed that this protein contains several structural domains, including a helix–loop–helix (HLH) domain located at the N-terminus and a coiled-coil (CC) domain at the C-terminus (Figure 4.2) [46]. In addition, the Act1 molecule possesses two TRAF interaction sequences: EEESE (residues 38–42) and EEERPA (residues 324–329) [48]. More recently, Act1 was also classified as a member of the SEFIR protein family, which comprises IL-17 receptors (IL-17Rs) and SEF proteins [49]. SEFIR proteins are characterized by the presence of a conserved sequence domain, the SEFIR domain, which shares similarity with the Toll/IL-1R (TIR) domain and is thought to be involved in homotypic interactions with other SEFIR/TIR-domain-containing proteins. Because of their close structural and functional similarities, the SEFIR and TIR domain-containing proteins have recently been united into a new STIR domain superfamily [49]. *Act1* is normally expressed in most tissues including the thymus, kidney, colon and placenta [46], but can be further induced in various cell types in response to extracellular stimuli through the activation of transcription factors including interferon regulatory factor-1 (IRF1) and CCAAT/enhancer-binding protein (C/EBP) [50].

Act1 inhibits systemic autoimmunity

Based on the potential role of Act1 in NF-κB regulation, *Act1*-deficient BALB/c mice were generated. Already at 3–4 weeks of age these mice showed signs of systemic autoimmunity including hypergammaglobulinaemia (IgGs, IgG1, IgG2a, IgG2b), hyper-IgE and inflammation around the eyes (Figure 4.3) [37,51]. Within a couple of months the mice start to express high levels of anti-nuclear autoantibodies including anti-double-stranded DNA and anti-total histones IgGs antibodies, as well as rheumatoid factor (Figure 4.4a). The production of autoantibodies in *Act1*-deficient mice is associated with splenomegaly, lymphodenopathy and B cell hyperplasia [37]. Further analysis revealed increased numbers of all mature B cell subsets (follicular, germinal centre, marginal zone and transitional B cells) but unaffected numbers of pre-pro, pro and immature B cells residing in the bone marrow. There was also no increase in the numbers of peripheral T cells. An increase in mature B cells has previously been

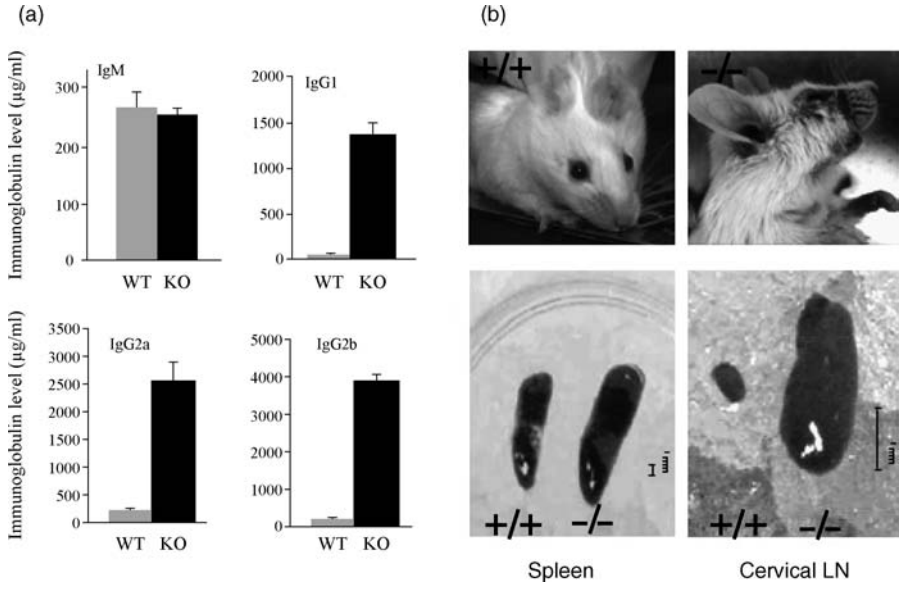

Figure 4.3 Early signs of autoimmunity in *Act1*-deficient mice. (a) Sera from 7-week-old Act1-deficient mice ($Act1^{-/-}$; KO) and wild-type littermates (WT) were tested for IgM, IgG1, IgG2a and IgG2b by ELISA. While levels of serum IgM were unchanged between the groups, *Act1*-deficient mice developed severe hypergammaglobulinaemia (IgG1, IgG2a, IgG2b). (b) The development of dry eyes (xerophthalmia) and skin lesions around the eyes started at 2 months of age. *Act1*-deficient mice show signs of infiltrations starting around 2 months of age. Shown are pictures of a 4–6-month-old wild-type mouse (top left panel) and of an age-matched *Act1*-deficient mouse (top right panel). Bottom panels: splenomegaly and lymphadenopathy was observed in *Act1*-deficient mice, but not in wild-type littermates [37].

reported in BAFF transgenic mice [52] and is generally associated with increased germinal centre formation and accumulation of antibody-secreting plasma cells. In correlation, *Act1*-deficient mice had higher numbers of germinal centres in lymph nodes as well as significantly more plasma cells [37]. Seven-to-twelve-month-old *Act1*-deficient mice developed lymphocyte infiltration of the kidney and IgGs–immune complex deposition in the glomeruli (although not proteinuria) (Figure 4.4b). Increased kidney infiltrations and immune complex deposition, without the development of kidney disease, has previously been reported in several mouse models of lupus [53–55].

The early symptoms of eye irritation are followed by severe eye inflammation and oral dryness, strikingly resembling the symptoms of xerophthalmia and xerostomia seen in patients with Sjögren's syndrome [56]. As the main characteristics of the disease are the production of anti-SSA/Ro and anti-SSB/La antibodies as well as reduced salivary gland function, *Act1*-deficient mice were tested and found positive for these features (Figure 4.5). Taken together, *Act1*-deficient mice were positive for the Sjögren's syndrome-associated phenotypes.

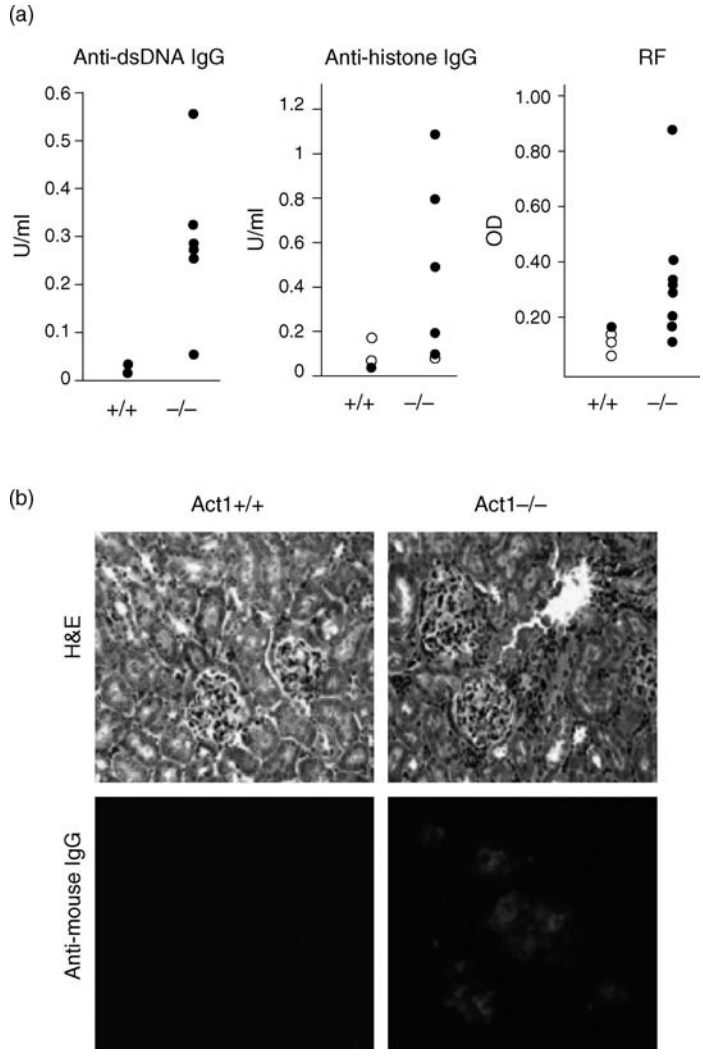

Figure 4.4 Autoantibody production and lupus-like disease in *Act1*-deficient mice. (a) Seven-month-old *Act1*-deficient ($-/-$) and wild-type mice ($+/+$) were bled and serum analysed for levels of anti-nuclear autoantibodies and rheumatoid factor (RF) by ELISA. (b) The presence of anti-nuclear autoantibodies was followed by lymphocytic kidney infiltrates and IgGs–immune complex deposition in the kidney glomeruli. Kidneys from 8-month-old *Act1*-deficient or wild-type littermates were stained with haematoxylin/eosin (H&E; top panel) or by fluorescence-conjugated anti-mouse IgGs (lower panel); 100× magnification [51]. dsDNA, double-stranded DNA.

In summary, Act1 deficiency leads to the development of systemic autoimmune disease as measured by peripheral B cell hyperplasia, hypergammaglobulinaemia, lymphocyte infiltration of the exocrine glands and the kidney, and elevated levels of serum anti-nuclear autoantibodies. As mentioned above, molecules known to affect B

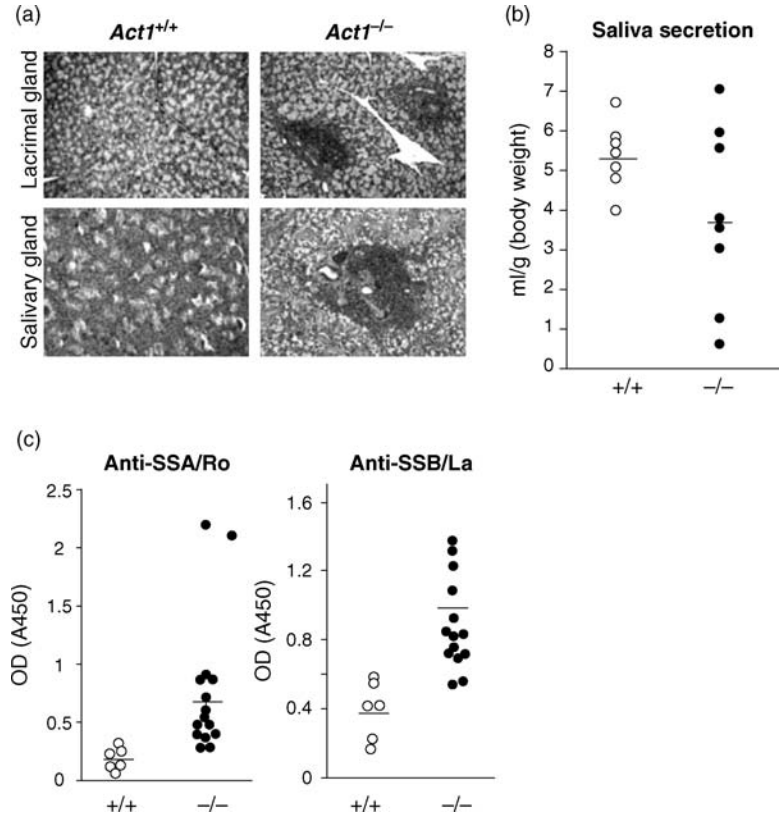

Figure 4.5 *Act1*-deficient mice develop Sjögren's-like disease. (a) Haematoxylin and eosin (H&E) staining of lacrimal and submaxillary (salivary) glands. Paraffin sections show massive lymphocyte infiltration in the glands of $Act1^{-/-}$, but not $Act1^{+/+}$ littermate control mice. All images are shown at a magnification of $\times 100$. (b) Reduced saliva secretion in 8–10-month-old *Act1*-deficient mice. The secretion of saliva was determined after pilocarpine stimulation and the values were normalized by body weight. (c) Sera from 7-month-old *Act1*-deficient and wild-type mice were analysed for the presence of anti-SSA/Ro and anti-SSB/La antibodies by ELISA [51].

cell differentiation and activation include BAFFR and CD40, both of which utilize TRAF proteins for downstream signalling [57–59]. The details on these pathways will be discussed in the following section.

Act1 inhibits excessive CD40 and BAFFR signalling

While *Act1*-deficient mice develop systemic autoimmunity associated with hyperactive B cells, similar pathologies were displayed by the B cell-specific *Act1*-deficient mice, implying a negative regulatory role of Act1 in B cell function [37]. It has been reported that endogenous Act1 is recruited to the BAFFR and CD40 in B cells upon stimulation

with BAFF and CD40L, respectively [37]. Once there, Act1 has been shown to interact strongly with TRAF3 (and weakly with TRAF2), suggesting that Act1 is involved in the CD40 and BAFFR signalling pathways. Importantly, CD40L- and BAFF-mediated survival is significantly increased in *Act1*-deficient B cells, with stronger IκBα phosphorylation, processing of NF-κB2 (p100) and activation of the c-Jun N-terminal kinase (JNK), extracellular-signal-regulated kinase (ERK) and p38 pathways, indicating that Act1 negatively regulates CD40L- and BAFF-mediated signalling events [37]. These findings demonstrate that Act1 plays an important role in the homeostasis of B cells by attenuating CD40 and BAFFR signalling.

The obvious next question is whether prolonged and increased CD40 and BAFFR signalling results in autoimmunity. To this extent, BAFF transgenic mice have been shown to develop elevated levels of anti-nuclear autoantibodies resembling lupus-like disease [60], whereas transgenic mice over-expressing CD40L in keratinocytes develop autoimmune dermatitis, lymphadenopathy, anti-double-stranded DNA antibodies and nephritis, which leads to renal failure [61]. If the negative regulatory effect of Act1 is absolutely dependent on either CD40 or BAFFR signalling, disease should be ameliorated in double-deficient mice ($Act1^{-/-}CD40^{-/-}$ or $Act1^{-/-}BAFF^{-/-}$). Interestingly, both $Act1^{-/-}CD40^{-/-}$ and $Act1^{-/-}BAFF^{-/-}$ strains retained some disease, including lymphocytic infiltration of the exocrine glands [37]. In contrast, B cell hyperplasia was only detectable in $Act1^{-/-}CD40^{-/-}$ mice, while anti-nuclear autoantibodies were only present in $Act1^{-/-}BAFF^{-/-}$ mice. These results indicate that Act1 modulates the survival of autoreactive B cells mainly through its negative regulatory role in BAFF-mediated cell survival, while the effect of Act1 on autoantibody production is probably through modulation of the CD40-mediated T cell-dependent antibody response. The impact of Act1 on both BAFF and CD40 pathways establishes *Act1*-deficient mice as a unique model to study distinct steps of autoimmunity and regulation of self-tolerance.

Model of Act1-controlled CD40 and BAFFR signalling

The exact mechanism by which Act1 exerts its inhibitory function on CD40 and BAFFR pathways is yet to be defined. However, the fact that *Act1* gene expression is induced following B cell stimulation with BAFF, CD40L or lipopolysaccharide may explain the important biological function of Act1 as a negative regulator. Thus, Act1 may provide a mechanism to control and dampen signalling involved in B cell survival and activation, and hence regulate B cell homeostasis. Likewise, TRAF2 and TRAF3 have been shown to function in a cooperative and non-redundant manner to suppress non-canonical NF-κB activation, gene expression and survival in mature B cells [62–65]. Since Act1 might be recruited to CD40 and BAFFR through its interaction with TRAFs, it is possible that TRAF2 and TRAF3 mediate signals to suppress CD40- and BAFFR-dependent signalling and cell survival through Act1. Importantly the result of Act1 dysregulation is increased NF-κB activation, which in turn affects the expression of a wide range of survival and activation factors.

Excessive expression of CD40- and BAFF-mediated genes results in the development of autoimmunity. To visualize the potential role of Act1 in B cell function, a working model has been proposed for CD40- and BAFFR-induced Act1-modulated signalling pathways (Figure 4.6).

Act1 is required for IL-17R signalling

The presence of a SEFIR domain in the C-terminal of Act1 (Figure 4.2) prompted the question of whether Act1 was involved in protein–protein interactions with SEFIR/TIR-domain-containing receptors, such as the IL-1R and IL-17R. To that end, co-immunoprecipitation assays clearly established that Act1 will indeed bind to the IL-17 receptor A (IL-17RA) in a transient manner after receptor ligation [38,66]. Using *Act1*-deficient mice, it was subsequently found that the IL-17-dependent expression of pro-inflammatory proteins such as IL-6, CXCL1 (KC), CXCL2 (MIP-2) and certain matrix metalloproteases (MMP3 and MMP9) absolutely required functional Act1 [38], proving that Act1 is a positive regulator of IL-17 signalling.

IL-17R ligation leads to activation of NF-κB [38]. If all IL-17R downstream signalling was dependent on NF-κB, stimulation of IKKα/β-deficient cells (unable to activate NF-κB) with IL-17 would result in a complete lack of target gene expression. This, however, is not the case as IL-17-induced intracellular signalling is only partially abolished in such cells [38]. Thus it is likely that Act1 is involved in other signalling pathways (in addition to NF-κB activation), including the activation of the transcription factors C/EBPβ and C/EBPδ. It is also interesting to note that many of the genes induced by IL-17 are regulated synergistically with other cytokines, such as TNF-α. However, the underlining molecular mechanisms of such synergy are still poorly understood.

Several reports have demonstrated that IL-17 induces pro-inflammatory gene expression not only at a transcriptional level but also at the post-transcriptional level by stabilizing cytokine and chemokine mRNAs. For example, it was demonstrated that Act1 is required for IL-17-induced mRNA stabilization of CXCL1 (KC) [67], suggesting a critical role of Act1 in post-transcriptional gene regulation.

Act1 mediates organ-specific autoimmunity

Anti-IL-17 antibody or neutralization by IL-17R–Fc attenuates the induction of chemokines, delays the onset of EAE and reverses the progression of EAE in the MOG-induced C57BL/6 (B6) model [68,69]. These previous studies suggested that IL-17 derived from infiltrating Th17 cells mediates the inflammatory responses in the central nervous system through the induction of chemokines, cytokines and MMPs. Given the positive effect of Act1 on IL-17R signalling, *Act1*-deficient mice were backcrossed to the B6 background and injected with MOG35–55 peptide to induce EAE. Act1-deficient mice developed significantly less severe and delayed-onset neurological disease (Figure 4.7a) [38]. As Act1 was found to be required for IL-17

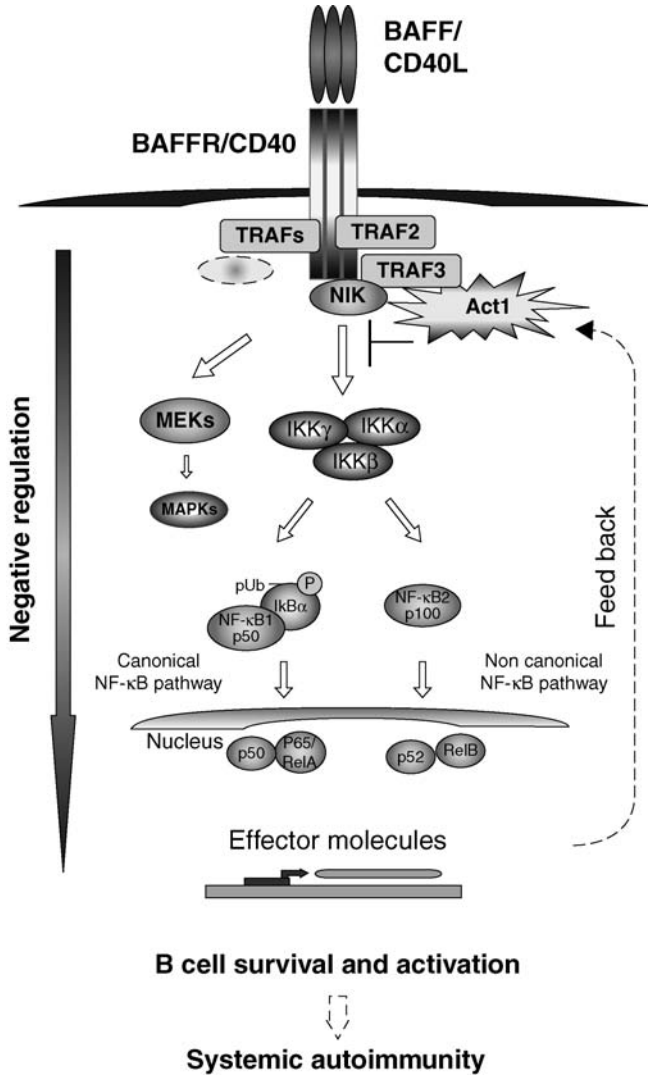

Figure 4.6 Act1 negatively regulates CD40 and BAFFR signalling. Ligation of BAFFR and CD40 leads to the recruitment of TRAF molecules and formation of an active signalling complex, resulting in activation of the mitogen-activated protein kinase (MAPK) pathway and activation of the IKK complex. The activation of IKK complex mediates the activation of the canonical NF-κB and non-canonical NF-κB pathways. These signalling events lead to upregulation of genes involved in B cell survival and activation. Act1 is recruited to BAFFR and CD40 through its interaction with TRAF3 and provides a negative signal that modulates downstream signalling. Upregulation of Act1 upon BAFF and CD40L stimulation may serve as a control mechanism to regulate the signalling (negative feedback). Act1 deficiency results in increased downstream signalling and excessive BAFF- and CD40-mediated B cell survival and activation. MEK, MAPK/ERK kinase.

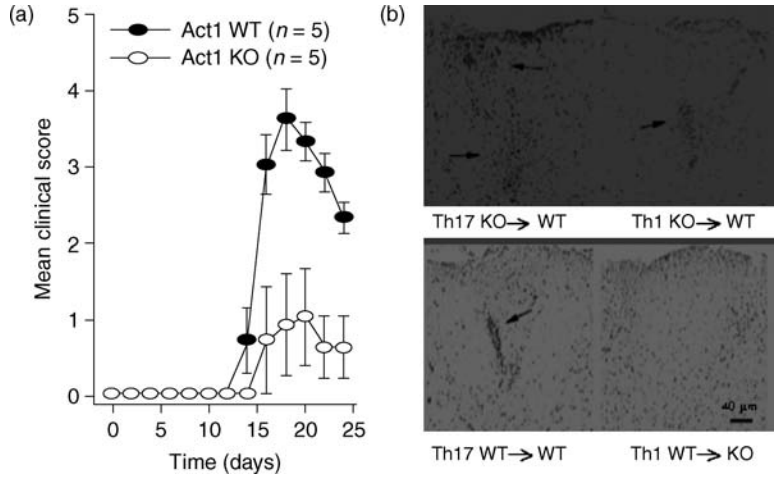

Figure 4.7 *Act1*-deficient mice develop significantly reduced EAE. (a) EAE was induced in wild-type (WT) and *Act1*-deficient (knockout, KO) mice by immunization with MOG35–55 peptide. Mean clinical scores were calculated daily for both groups of mice; $P < 0.0001$. (b) Histological analysis of spinal cords from wild-type mice that received Th1 or Th17 cells from *Act1*-deficient mice. Twenty days later, recipients were killed and sections were stained with haematoxylin/eosin. Th17 cells from *Act1*-deficient mice were fully functional in passive transfer of disease to wild-type recipients [38]. Arrows denote areas of mononuclear cell infiltration.

signalling *in vitro* and *ex vivo*, the lesser EAE pathogenesis observed in *Act1*-deficient mice was probably due to the lack of IL-17 signalling. In this regard, it is worth mentioning that the effect of inhibited IL-17R signalling was clearly not an effect of altered Th17 cell function as Th17 cells isolated from MOG-treated *Act1*-deficient mice could passively transfer disease into wild-type mice (Figure 4.7b). More likely, the effect was due to the lack of IL-17 responsiveness by astrocytes expressed in the central nervous system [70], as astrocytes previously have been found to express the IL-17R [38].

Model of Act1-controlled IL-17R signalling

Upon IL-17 stimulation, Act1 is recruited to the IL-17R (see the model in Figure 4.8). While Act1 interacts well with the full-length IL-17R, much less Act1 is associated with the truncated IL-17R (ΔSEFIR) [38], indicating that the recruitment of Act1 to the IL-17R is dependent on SEFIR–SEFIR domain interactions. TRAF6 and TRAF3 are also recruited to the IL-17 receptor, but in a SEFIR-domain-independent manner [38,71,72]. Whereas TRAF6 has been clearly shown to play a critical role in IL-17-mediated NF-κB activation, the function of TRAF3 in IL-17 signalling remains unclear [71,72]. The fact that endogenous transforming growth factor β (TGF-β)-activated kinase l (TAK1) interacts with Act1 in response to IL-17 stimulation may suggest that TAK1 is involved in IL-17 signalling [38]. In support of this, IL-17-induced

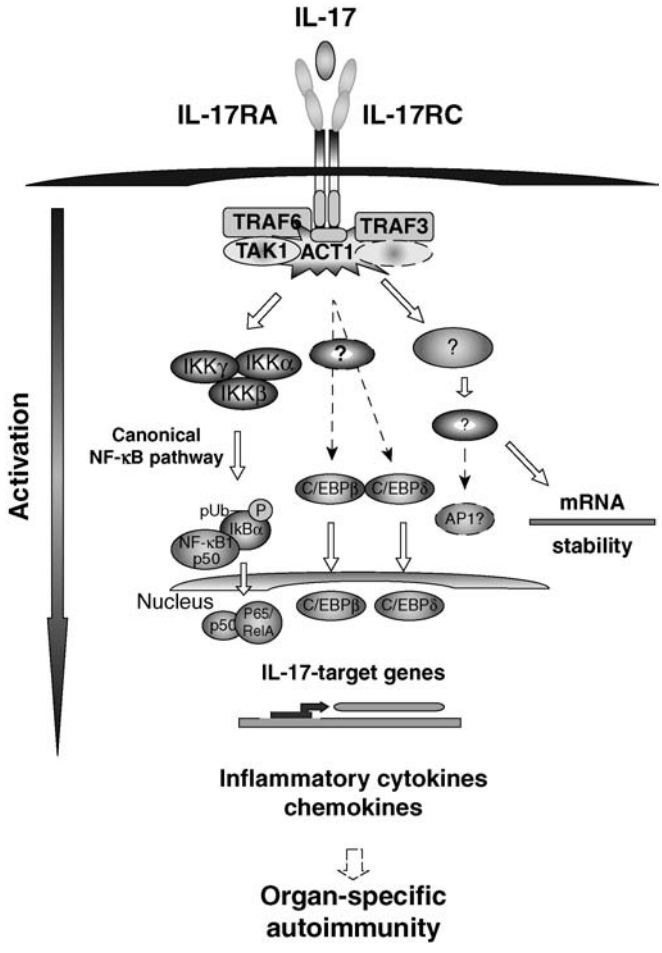

Figure 4.8 IL-17R signalling requires Act1. The IL-17R is a heteromeric receptor, composed of IL-17RA and IL-17RC subunits. Binding of IL-17 leads to the recruitment of Act1 to the IL-17R, which is mediated by a direct SEFIR–SEFIR interaction. The binding of Act1 to the IL17R leads to further recruitment of TRAF6, TRAF3 and TGF-β-activated kinase l (TAK1) into the signalling complex and subsequent activation of the canonical NF-κB pathway, activator protein 1 (AP-1) pathway and activation of C/EBPβ and C/EBPδ transcription factors. The activation of these signalling pathways leads to regulation of the expression of pro-inflammatory genes at both transcriptional and post-transcriptional (mRNA stability) levels. IL-17-induced expression of pro-inflammatory genes is abolished in the absence of Act1.

CXCL1 (KC) production is partially reduced in TAK1-deficient mouse embryonic fibroblasts. Since TAK1 has been shown to function as an intermediate signalling component in TIR-induced NF-κB and JNK activation, it is likely that TAK1 plays a similar role in mediating IL-17-induced Act1-dependent NF-κB and JNK activation. Finally, Act1 is also required for IL-17-induced expression of the transcription factor C/EBP and IL-17-mediated mRNA stabilization [38,66]; however, the components

downstream of Act1 for these signalling pathways remain to be identified. To summarize these findings a model for IL-17-induced Act1-mediated pathways has been proposed (Figure 4.8).

4.5 Conclusions

Few genes have been identified that may differentially control systemic and organ-specific autoimmunity. We propose that dysregulation (gain or loss of function) of *Act1* may in fact lead to autoimmunity. Whereas Act1 could drive organ-specific autoimmunity with gain-of-function mutations, it also can cause systemic autoimmunity with loss-of-function mutations. Dysregulation of Act1 could result in alteration of the expression of multiple genes through either a direct or indirect effect on the activity of different transcription factors as well as its impact on mRNA stability.

Autoimmunity in humans is heavily dependent on the genetic predisposition of the individual as well as on the effect of environmental factors (Figure 4.9). Based on

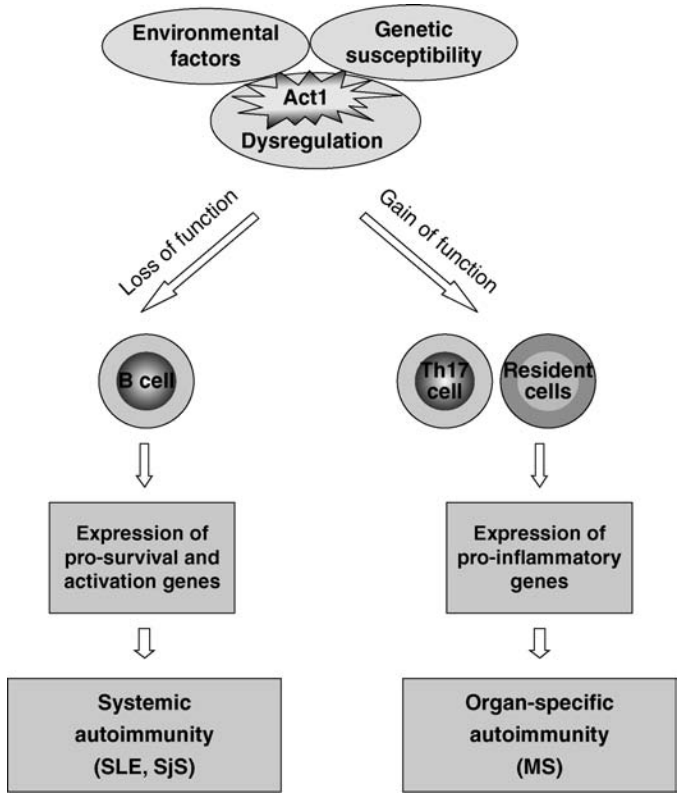

Figure 4.9 Model of the regulatory function of Act1. Dysregulation of Act1 in genetically susceptible individuals could be a contributing factor in the development of systemic as well as organ-specific autoimmunity. MS, multiple sclerosis; SjS, Sjögren's syndrome.

the functions of Act1 on B cell activation and survival (CD40 and BAFFR signalling) and on IL-17-mediated signalling, it is tempting to suggest that Act1 may have a critical function in balancing B and T cell activation and survival. This view fits well with the general dogma that dysregulation in the lymphocyte compartment is often found in autoimmune patients and in genetically engineered mouse models of autoimmune diseases.

References

1. Rudofsky, U.H. and Lawrence, D.A. (1999) New Zealand mixed mice: a genetic systemic lupus erythematosus model for assessing environmental effects. *Environmental Health Perspectives* **107** (suppl 5), 713–721.
2. Kotzin, B.L. and Palmer, E. (1987) The contribution of NZW genes to lupus-like disease in (NZB × NZW)F1 mice. *Journal of Experimental Medicine* **165**, 1237–1251.
3. Kono, D.H., Burlingame, R.W., Owens, D.G. *et al.* (1994) Lupus susceptibility loci in New Zealand mice. *Proceedings of the National Academy of Sciences, USA* **91**, 10168–10172.
4. Rozzo, S.J., Vyse, T.J., Drake, C.G. and Kotzin, B.L. (1996) Effect of genetic background on the contribution of New Zealand black loci to autoimmune lupus nephritis. *Proceedings of the National Academy of Sciences, USA* **93**, 15164–15168.
5. Mohan, C., Alas, E., Morel, L. *et al.* (1998) Genetic dissection of SLE pathogenesis. Sle1 on murine chromosome 1 leads to a selective, loss of tolerance to H2A/H2B/DNA subnucleosomes. *Journal of Clinical Investigation* **101**, 1362–1372.
6. Andrews, B.S., Eisenberg, R.A., Theofilopoulos, A.N. *et al.* (1978) Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestations in several strains. *Journal of Experimental Medicine* **148**, 1198–1215.
7. Murphy, E.D. and Roths, J.B. (1978) Autoimmunity and lymphoproliferation In: Rose, N.R., Bigazzi, P.E., Warner, N.L. (eds), *Genetic Control of Autoimmune Disease*, pp. 207–220. Elsevier/North Holland Biomedical Press, Amsterdam.
8. Tabbara, K.F. and Vera-Cristo, C.L. (2000) Sjogren syndrome. *Current Opinion in Ophthalmology* **11**, 449–454.
9. Steinman, L. (1996) Multiple sclerosis: a coordinated immunological attack against myelin in the central nervous system. *Cell* **85**, 299–302.
10. Gold, R., Linington, C. and Lassmann, H. (2006) Understanding pathogenesis and therapy of multiple sclerosis via animal models: 70 years of merits and culprits in experimental autoimmune encephalomyelitis research. *Brain* **129**, 1953–1971.
11. Linthicum, D.S. (1982) Development of acute autoimmune encephalomyelitis in mice: factors regulating the effector phase of the disease. *Immunobiology* **162**, 211–220.
12. Batten, M., Groom, J., Cachero, T.G. *et al.* (2000) BAFF mediates survival of peripheral immature B lymphocytes. *Journal of Experimental Medicine* **192**, 1453–1466.
13. Schiemann, B., Gommerman, J.L., Vora, K. *et al.* (2001) An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science* **293**, 2111–2114.
14. Mackay, F., Schneider, P., Rennert, P. and Browning, J. (2003) BAFF AND APRIL: a tutorial on B cell survival. *Annual Reviews in Immunology* **21**, 231–264.
15. Banchereau, J., Bazan, F., Blanchard, D. *et al.* (1994) The CD40 antigen and its ligand. *Annual Reviews in Immunology* **12**, 881–922.
16. Mackay, F., Woodcock, S.A., Lawton, P. *et al.* (1999) Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *Journal of Experimental Medicine* **190**, 1697–1710.
17. Mehling, A., Loser, K., Varga, G. *et al.* (2001) Overexpression of CD40 ligand in murine epidermis results in chronic skin inflammation and systemic autoimmunity. *Journal of Experimental Medicine* **194**, 615–628.

18. Pers, J.O., Daridon, C., Devauchelle, V. *et al.* (2005) BAFF overexpression is associated with autoantibody production in autoimmune diseases. *Annals of the New York Academy of Sciences* **1050**, 34–39.
19. DiDonato, J.A., Hayakawa, M., Rothwarf, D.M. *et al.* (1997) A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature* **388**, 548–554.
20. Mercurio, F., Zhu, H., Murray, B.W. *et al.* (1997) IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science* **278**, 860–866.
21. Regnier, C.H., Song, H.Y., Gao, X. *et al.* (1997) Identification and characterization of an IkappaB kinase. *Cell* **90**, 373–383.
22. Woronicz, J.D., Gao, X., Cao, Z. *et al.* (1997) IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK. *Science* **278**, 866–869.
23. Zandi, E., Rothwarf, D.M., Delhase, M. *et al.* (1997) The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. *Cell* **91**, 243–252.
24. Pasparakis, M., Schmidt-Supprian, M. and Rajewsky, K. (2002) IkappaB kinase signaling is essential for maintenance of mature B cells. *Journal of Experimental Medicine* **196**, 743–752.
25. Li, Z.W., Omori, S.A., Labuda, T. *et al.* (2003) IKK beta is required for peripheral B cell survival and proliferation. *Journal of Immunology* **170**, 4630–4637.
26. Kaisho, T., Takeda, K., Tsujimura, T. *et al.* (2001) IkappaB kinase alpha is essential for mature B cell development and function. *Journal of Experimental Medicine* **193**, 417–426.
27. Coope, H.J., Atkinson, P.G., Huhse, B. *et al.* (2002) CD40 regulates the processing of NF-kappaB2 p100 to p52. *EMBO Journal* **21**, 5375–5385.
28. Senftleben, U., Cao, Y., Xiao, G. *et al.* (2001) Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. *Science* **293**, 1495–1499.
29. Ghosh, S. and Karin, M. (2002) Missing pieces in the NF-kappaB puzzle. *Cell* **109**(suppl.), S81–S96.
30. Claudio, E., Brown, K., Park, S. *et al.* (2002) BAFF-induced NEMO-independent processing of NF-kappa B2 in maturing B cells. *Nature Immunology* **3**, 958–965.
31. Franzoso, G., Carlson, L., Poljak, L. *et al.* (1998) Mice deficient in nuclear factor (NF)-kappa B/p52 present with defects in humoral responses, germinal center reactions, and splenic microarchitecture. *Journal of Experimental Medicine* **187**, 147–159.
32. Kayagaki, N., Yan, M., Seshasayee, D. *et al.* (2002) BAFF/BLyS receptor 3 binds the B cell survival factor BAFF ligand through a discrete surface loop and promotes processing of NF-kappaB2. *Immunity* **17**, 515–524.
33. Agarwal, S., Misra, R. and Aggarwal, A. (2008) Interleukin 17 levels are increased in juvenile idiopathic arthritis synovial fluid and induce synovial fibroblasts to produce proinflammatory cytokines and matrix metalloproteinases. *Journal of Rheumatology* **35**, 515–519.
34. Iwakura, Y. and Ishigame, H. (2006) The IL-23/IL-17 axis in inflammation. *Journal of Clinical Investigation* **116**, 1218–1222.
35. Lubberts, E., Schwarzenberger, P., Huang, W. *et al.* (2005) Requirement of IL-17 receptor signaling in radiation-resistant cells in the joint for full progression of destructive synovitis. *Journal of Immunology* **175**, 3360–3368.
36. Zhang, Z., Zheng, M., Bindas, J. *et al.* (2006) Critical role of IL-17 receptor signaling in acute TNBS-induced colitis. *Inflammatory Bowel Diseases* **12**, 382–388.
37. Qian, Y., Qin, J., Cui, G. *et al.* (2004) Act1, a negative regulator in CD40- and BAFF-mediated B cell survival. *Immunity* **21**, 575–587.
38. Qian, Y., Liu, C., Hartupee, J. *et al.* (2007) The adaptor Act1 is required for interleukin 17-dependent signaling associated with autoimmune and inflammatory disease. *Nature Immunology* **8**, 247–256.
39. Watanabe-Fukunaga, R., Brannan, C.I., Copeland, N.G. *et al.* (1992) Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* **356**, 314–317.
40. Sabelko, K.A., Kelly, K.A., Nahm, M.H. *et al.* (1997) Fas and Fas ligand enhance the pathogenesis of experimental allergic encephalomyelitis, but are not essential for immune privilege in the central nervous system. *Journal of Immunology* **159**, 3096–3099.

41. Roths, J.B., Murphy, E.D. and Eicher, E.M. (1984) A new mutation, gld, that produces lymphoproliferation and autoimmunity in C3H/HeJ mice. *Journal of Experimental Medicine* **159**, 1–20.
42. Nishimura, H., Nose, M., Hiai, H. *et al.* (1999) Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* **11**, 141–151.
43. Salama, A.D., Chitnis, T., Imitola, J. *et al.* (2003) Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis. *Journal of Experimental Medicine* **198**, 71–78.
44. Fujimoto, M., Tsutsui, H., Xinshou, O. *et al.* (2004) Inadequate induction of suppressor of cytokine signaling-1 causes systemic autoimmune diseases. *International Immunology* **16**, 303–314.
45. Tanaka, K., Ichiyama, K., Hashimoto, M. *et al.* (2008) Loss of suppressor of cytokine signaling 1 in helper T cells leads to defective Th17 differentiation by enhancing antagonistic effects of IFN-gamma on STAT3 and Smads. *Journal of Immunology* **180**, 3746–3756.
46. Li, X., Commane, M., Nie, H. *et al.* (2000) Act1, an NF-kappa B-activating protein. *Proceedings of the National Academy of Sciences, USA* **97**, 10489–10493.
47. Leonardi, A., Chariot, A., Claudio, E. *et al.* (2000) CIKS, a connection to Ikappa B kinase and stress-activated protein kinase. *Proceedings of the National Academy of Sciences, USA* **97**, 10494–10499.
48. Qian, Y., Zhao, Z., Jiang, Z. and Li, X. (2002) Role of NF kappa B activator Act1 in CD40-mediated signaling in epithelial cells. *Proceedings of the National Academy of Sciences, USA* **99**, 9386–9391.
49. Novatchkova, M., Leibbrandt, A., Werzowa, J. *et al.* (2003) The STIR-domain superfamily in signal transduction, development and immunity. *Trends in Biochemical Sciences* **28**, 226–269.
50. Zhao, Z., Qian, Y., Wald, D. *et al.* (2003) IFN regulatory factor-1 is required for the up-regulation of the CD40-NF-kappa B activator 1 axis during airway inflammation. *Journal of Immunology* **170**, 5674–5680.
51. Qian, Y., Giltiay, N., Xiao, J. *et al.* (2008) Deficiency of Act1, a critical modulator of B cell function, leads to development of Sjögren's syndrome. *European Journal of Immunology* **38**, 2219–2228.
52. Mackay, F., Woodcock, S.A., Lawton, P. *et al.* (1999) Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *Journal of Experimental Medicine* **190**, 1697–1710.
53. Wakeland, E.K., Liu, K., Graham, R.R. and Behrens, T.W. (2001) Delineating the genetic basis of systemic lupus erythematosus. *Immunity* **15**, 397–408.
54. Rozzo, S.J., Allard, J.D., Choubey, D. *et al.* (2001) Evidence for an interferon-inducible gene, Ifi202, in the susceptibility to systemic lupus. *Immunity* **15**, 435–443.
55. Stohl, W., Xu, D., Kim, K.S. *et al.* (2005) BAFF overexpression and accelerated glomerular disease in mice with an incomplete genetic predisposition to systemic lupus erythematosus. *Arthritis & Rheumatism* **52**, 2080–2091.
56. Fox, P.C. (2007) Autoimmune diseases and Sjogren's syndrome: an autoimmune exocrinopathy. *Annals of the New York Academy of Sciences* **1098**, 15–21.
57. Bossen, C. and Schneider, P. (2006) BAFF, APRIL and their receptors: structure, function and signaling. *Seminars in Immunology* **18**, 263–275.
58. Brink, R. (2006) Regulation of B cell self-tolerance by BAFF. *Seminars in Immunology* **18**, 276–283.
59. Bishop, G.A., Moore, C.R., Xie, P. *et al.* (2007) TRAF proteins in CD40 signaling. *Advances in Experimental Medicine and Biology* **597**, 131–151.
60. Mackay, F., Woodcock, S.A., Lawton, P. *et al.* (1999) Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *Journal of Experimental Medicine* **190**, 1697–1710.
61. Mehling, A., Loser, K., Varga, G. *et al.* (2001) Overexpression of CD40 ligand in murine epidermis results in chronic skin inflammation and systemic autoimmunity. *Journal of Experimental Medicine* **194**, 615–628.

62. Xie, P., Stunz, L.L., Larison, K.D. *et al.* (2007) Tumor necrosis factor receptor-associated factor 3 is a critical regulator of B cell homeostasis in secondary lymphoid organs. *Immunity* **27**, 253–267.
63. Bishop, G.A. and Xie, P. (2007) Multiple roles of TRAF3 signaling in lymphocyte function. *Immunologic Research* **39**, 22–32.
64. Xu, L.G. and Shu, H.B. (2002) TNFR-associated factor-3 is associated with BAFF-R and negatively regulates BAFF-R-mediated NF-kappa B activation and IL-10 production. *Journal of Immunology* **169**, 6883–6889.
65. Hauer, J., Puschner, S., Ramakrishnan, P. *et al.* (2005) TNF receptor (TNFR)-associated factor (TRAF) 3 serves as an inhibitor of TRAF2/5-mediated activation of the noncanonical NF-kappaB pathway by TRAF-binding TNFRs. *Proceedings of the National Academy of Sciences, USA* **102**, 2874–2879.
66. Chang, S.H., Park, H. and Dong, C. (2006) Act1 adaptor protein is an immediate and essential signaling component of interleukin-17 receptor. *Journal of Biological Chemistry* **281**, 35603–35607.
67. Hartupee, J., Liu, C., Novotny, M. *et al.* (2007) IL-17 enhances chemokine gene expression through mRNA stabilization. *Journal of Immunology* **179**, 4135–4141.
68. Hofstetter, H.H., Ibrahim, S.M., Koczan, D. *et al.* (2005) Therapeutic efficacy of IL-17 neutralization in murine experimental autoimmune encephalomyelitis. *Cellular Immunology* **237**, 123–130.
69. Park, H., Li, Z., Yang, X.O. *et al.* (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nature Immunology* **6**, 1133–1141.
70. Dong, Y. and Benveniste, E.N. (2001) Immune function of astrocytes. *Glia* **36**, 180–190.
71. Yao, Z., Spriggs, M.K., Derry, J.M. *et al.* (1997) Molecular characterization of the human interleukin (IL)-17 receptor. *Cytokine* **9**, 794–800.
72. Schwandner, R., Yamaguchi, K. and Cao, Z. (2000) Requirement of tumor necrosis factor receptor-associated factor (TRAF)6 in interleukin 17 signal transduction. *Journal of Experimental Medicine* **191**, 1233–1240.

Abstract

Autoimmune diseases generally occur in genetically susceptible individuals. Although most cases of disease seem to be a result of multiple genes, occasional single-gene polymorphisms may cause disease development. Act1 is a modulator of both the adaptive and innate immune responses, and tight regulation of Act1 is critical for the control of autoimmune development in mice. Thus, whereas Act1 inhibits spontaneous lupus-like disease, it promotes induced experimental autoimmune encephalomyelitis in mouse models. In this chapter we describe the outcome of dysregulated Act1 and some of the potential mechanisms by which this may lead to autoimmunity.

Key words: Act1; autoimmunity; B cell; BAFF; IL-17 receptor; SLE; Sjögren's syndrome; multiple sclerosis; NF-κB

5

Regulation of T cell anergy and escape from regulatory T cell suppression by Cbl-b

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5.1 Introduction

The key function of the immune system is the discrimination between self, altered self and non-self to protect from invading pathogens or aberrant outgrowth of tumour cells while, at the same time preventing autoaggressive attacks against healthy self tissue. Therefore, T cell activation or induction of T cell anergy and tolerance have to be tightly controlled processes. On the molecular level multiple pathways have been implicated in the control of co-stimulation and the induction of T cell tolerance. Recent publications reported the important role of functioning of the ubiquitin E3 ligase Cbl-b, a member of the the family of the Cbl (Casitas B-lineage lymphoma) proteins, as an intrinsic mediator of T cell anergy *in vitro* and *in vivo*, maintaining the balance between tolerance, activation and autoimmunity [1,2].

5.2 Mechanisms of T cell tolerance induction

To prevent harmful self destruction, tolerance is achieved at several different levels during T cell development and peripheral T cell activation. In the thymus the majority of thymocytes that carry antigen receptors with high affinity and avidity for self-antigens that would mature into autoreactive T cells are deleted by negative selection [3–6]. However, central tolerance is incomplete and autoreactive T cells can

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escape from the thymic milieu [7], which explains the need for additional peripheral tolerance mechanisms. Peripheral T cell tolerance includes dominant immunosuppression mediated by regulatory T cells (Tregs), ignorance to antigens expressed at low levels or at immune-privileged sites, cell-intrinsic functional inactivation (anergy) or deletion of activated T cells through activation-induced cell death [8–10]. In addition, the activation and differentiation status of antigen-presenting cells depending on the tissue and the inflammatory environment as well as the strength of T cell receptor (TCR)– major histocompatibility complex (MHC)-peptide interactions and the developmental state of the T cells influence the outcome of the response [11].

Dominant suppression in the periphery is mediated by suppressor T cells or Tregs. Various subtypes have been described in the recent years including the best-characterized, so-called natural Tregs originating from the thymus as well as peripherally induced so-called adaptive Treg populations [12]. Natural Tregs are a specific subset of $CD4^+$ T cells expressing the Treg-specific transcription factor FoxP3 [13,14] as well as the interleukin (IL)-2 receptor α chain CD25 [15], CD62L [16] and specific CD45 isoforms [17]. They play an essential role in immune homeostasis since ablation of Tregs in mice as well as mutations of FoxP3 in mice and in humans result in the development of severe multi-organ autoimmune disease [18]. Other suppressor cells include $CD4^+$ IL-10-producing Tr1 cells [19] and transforming growth factor β (TGF-β)-producing T helper type 3 cells [20], as well as $CD8^+$ suppressor T cells [21] and NKT cells and $\gamma:\delta$ T cell subtypes [22–24] of which some, but not all, express FoxP3. Multiple *in vitro* as well as *in vivo* studies have proposed a variety of Treg suppression mechanisms acting directly on T cells or on antigen-presenting cell activation, differentiation and survival (summarized in [25]). This supposedly depends on the nature of the immune response, the causing agent, the tissue environment and the immune status of the individual. Once activated Tregs can suppress T cell proliferation in an antigen-non-specific fashion called bystander suppression. In particular, experimental systems *in vivo* have shown that Tregs can further create a milieu which promotes the differentiation of new Treg populations from naïve effector cells with distinct antigen specificity within the tissue context, a process termed infectious tolerance (summarized in [25]).

In various *in vitro* as well as *in vivo* models T cell intrinsic unresponsiveness is induced by specific stimulation protocols. In such systems, T cell clonal anergy is defined as a state in which T cells are functionally inactive following an antigen encounter, but remain viable for an extended period of time in this hypo-responsive state [26]. Maintenance of T cells in the anergic state can be dependent on antigen persistence and can be reversed by the addition of exogenous IL-2 depending on the particular model [27–29].

The common paradigm of antigen-specific T cell activation proposes the requirement for at least two signals to induce effector responses: the antigen-specific signal via the TCR recognizing its cognate peptide bound to an MHC molecule (signal 1) and a co-stimulatory signal (signal 2) primarily provided by activated professional antigen-presenting cells. For the of priming of naïve T cells, CD28 co-stimulation by engagement to B7 ligands on antigen-presenting cells has been identified as a key molecule

involved in T cell co-stimulation [30,31]. This co-stimulatory signal enhances TCR proximal signalling [32], survival by induction of pro-survival proteins such as Bcl-x_L [33,34], and cytokine production such as IL-2 [35] (Figure 5.1). Importantly, CD28-mediated signalling leads to cytoskeleton rearrangements which are essential for immune synapse formation and therefore proper activation [29,36–40]. Additionally, $CD8^+$ T cells may require cytokines (signal 3) to become fully activated and develop effector functions. Signal 3 can be provided by IL-12 [41] or type I interferons [42]. The strength of signal 1, the peptide–MHC–TCR interaction, is further crucial in determining the outcome of T cell activation. Whereas TCR stimulation in the presence of co-stimulation requires a certain peptide/TCR affinity threshold to produce proper T cell activation, TCR stimulation below this threshold with a

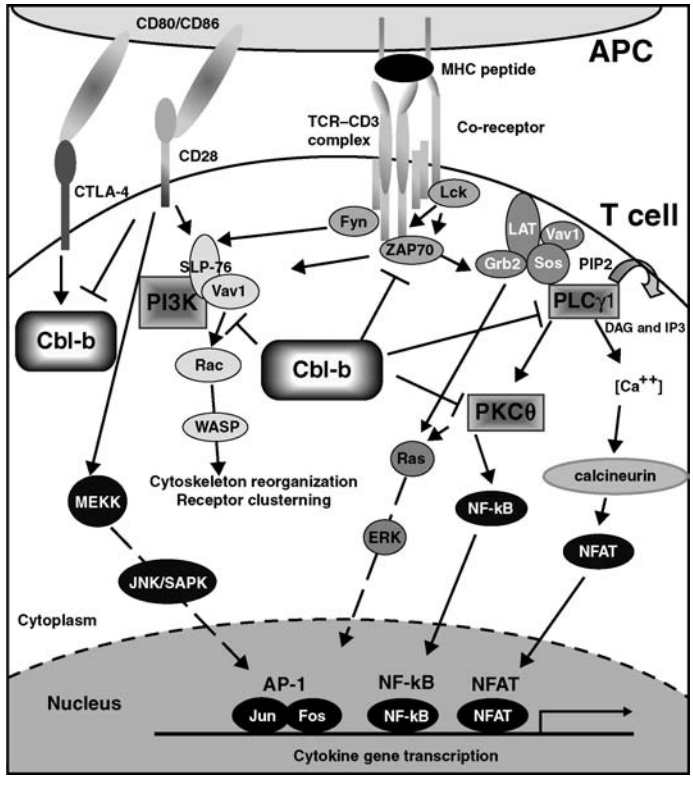

Figure 5.1 Schematic representation of TCR- and CD28-mediated signalling pathways. Productive T cell stimulation by both the TCR and the co-stimulatory receptor CD28 leads to the activation of proximal signalling pathways. Cbl-b functions as a negative regulator of T cell activation, interfering with crucial mediators like PI3K, Vav1 and PKCθ and which can be bypassed by CD28 co-stimulation. Cbl-b itself is negatively regulated by CD28, whereas the inhibitory receptor CTLA-4 positively regulates Cbl-b expression. The signalling cascades and interactions are simplified and do not contain all molecules involved. For further details please see text. APC, antigen-presenting cell; DAG, diacylglycerol; IP3, inositol 3-phosphate; LAT, linker for activation of T cell; MEKK, MAPK kinase; SAPK, stress-activated protein kinase; WASP, Wiskott–Aldrich syndrome protein.

low-ffinity ligand or strong TCR stimulation in the absence of co-stimulation has been shown to result in anergy [43–45].

5.3 Molecular establishment of T cell anergy

Depending on the model of anergy induction multiple changes in TCR downstream signalling have been reported, which can differ depending on the conditions [46]. Multiple alterations in TCR proximal signalling, such as defective $p56^{Lck}$ and Zap70 activation, preferential activation of $p59^{fyn}$ and Rap1 [47] and defects in Ras-mediated extracellular-signal-regulated kinase (ERK) phosphorylation, result in the induction of an unresponsive, anergic state [48–51]. The observation that anergic T cells display a defect in IL-2 production led to the discovery that altered signalling in anergic T cells results in reduced activator protein 1 (AP-1) binding. Consequently, reduced enzymic activity of the AP-1-activating upstream mitogen-activated protein kinases (MAPKs) c-Jun N-terminal kinase (JNK) and ERK, as well as defective activation of the GTPase Ras in anergic T cells, have been reported [48–50]. Diacylglycerol kinases (DGKs) were recently identified as an upstream negative regulator of Ras in T cell anergy [52,53]. dgk-$\alpha^{-/-}$ as well as dgk-$\zeta^{-/-}$ T cells show increased Ras activity and enhanced ERK and JNK phosphorylation and dgk-$\alpha^{-/-}$ mice display impaired angery induction in response to superantigen *in vivo* [53,54]. Mechanistically, DGK-α inhibits membrane translocation of the Ras guanine nucleotide-exchange factor RasGRP1 which results in decreased Ras-GTP and reduced Ras activation [52]. Inhibition of AP-1 via negative regulation of Ras might also be essential for maintenance of the anergic state, since introduction of a constitutive active form of Ras into anergic T cells restores IL-2 production and antigen-induced proliferation [52]. On the other hand, there are reports showing that anergy can be induced in T cells transfected with constitutively active Ras [55,56].

IL-2 has been shown to be crucial for cell-cycle entry but also cell-cycle progression from the G_1 to the S phase has been determined as another checkpoint driving T cells towards activation or anergy. Blockade of cell-cycle progression during anergy induction is achieved by downregulation of the cyclin-dependent kinase (CDK) inhibitor $p27^{kip1}$ leading to CDK-mediated phosphorylation of the transcription factors Smad2, Smad3 and retinoblastoma (Rb) protein via the CD28 co-stimulatory pathway and IL-2. It has been shown that $p27^{kip1}$-deficient cells are resistant to tolerance induction [57] and $p27^{kip1}$ is upregulated in tolerant T cell clones [58]. Additionally a recent *in vivo* study illustrates that T cells fail to downregulate the CDK inhibitor $p27^{kip1}$ under conditions of suboptimal activation such as *in vivo* blocking of CD28 and CD40, resulting in a failure of Smad3 phosphorylation and induction of tolerance [59].

TCR-only stimulation in the absence of CD28–CD80/CD86 interactions triggers calcium-activated signalling pathways via calmodulin/calcineurin but fails to fully activate other pathways like the Ras/MAPK pathway as mentioned above, or the protein kinase C (PKC) and the inhibitor of nuclear factor κB (NF-κB) kinase (IKK) pathways. Calcium-mediated signalling activation of nuclear factor of activated T cells (NFAT) in

the absence of AP-1 proteins and possibly other transcriptional partners directs the transactivation of a specific programme of genes encoding proteases, tyrosine phosphatases or transcriptional repressors to induce and maintain an anergic state [1,10,60,61]. In this ionomycine-induced tolerance system, the E3 ligases Itch, gene related to anergy in lymphocytes (GRAIL) and Cbl-b were identified as target genes of NFAT-regulated immunotolerance [1]. The E3 ligase Cbl-b has been described as an important intracellular regulator setting the activation threshold of the antigen receptor and as a negative regulator of CD28 co-stimulation [1,2,62–65].

5.4 Ubiquitin E3 ligases in T cell tolerance

Protein ubiquitylation is a conserved process in which the 76-amino-acid peptide ubiquitin is transferred to target proteins thereby regulating protein stability, localization and function. Ubiquitin modification of proteins is mediated by a protein cascade consisting of three classes of enzyme, termed E1, E2 and E3. In the initial activating step, the E1 enzyme forms a thiol ester bond with the C-terminal glycine residue of ubiquitin followed by ubiquitin transfer to the ubiquitin-conjugating (Ubc) E2 enzymes. In a final step, the E3 ligase serves as a platform binding the E2–ubiquitin complex as well as the substrate and catalyses the formation of an isopeptide bond between ubiquitin and the ε-amino group of a specific lysine residue on the substrate [66]. Depending on the context only one ubiquitin is transferred or a polyubiquitin chain is attached to the target protein. Mono-ubiquitination or poly-ubiquitination via lysines other then residue 48 have been shown to alter protein trafficking or function while poly-ubiquitination via residue 48 tags substrate protein to the classical degradation pathway via the 26S proteasome [67,68]. The specificity of the process is determined by different E3 ligases interacting with their substrates. This explains why a high number of E3 ligases in contrast to only one E1 enzyme and few ubiquitous E2 enzymes have been found in the genome.

Two families of E3 ligase have been described according to their structure and mode of action: the HECT (homologous to E6-associated protein carboxyl terminus) domain containing E3 ligases and E3 ligases harbouring a RING (Really Interesting New Gene) domain motif [69]. A conserved active cysteine residue located in the C-terminus of the HECT domain catalyses ubiquitin transfer to the substrate. For substrate binding several members of the HECT-type E3 ligases contain an N-terminal Ca^{2+}-binding PKC-related C2 domain followed by multiple WW domains. The RING finger motif containing E3 ligases can be further subdivided into single protein E3 and multi-subunit E3 ligases. The RING finger-type E3 ligases promote the transfer of ubiquitin from E2 enzyme to the specific target protein, but do not bind ubiquitin directly [69].

Members of both E3 ligases families, the HECT (Itch and NEDD4) and RING (Cbl-b and GRAIL) types, have been implicated in peripheral T cell tolerance [1,2,62,63,70–72]. The HECT-type E3 ligase Itch is upregulated upon anergizing stimuli and localizes predominantly to endocytic vesicles [1,73–75]. Initial genetic inactivation of Itch in

mice resulted in the development of a characteristic severe autoimmune dermatitis-type disorder accompanied by constant scratching of the skin [72,76]. Functional analysis of *itch*$^{-/-}$ T cells revealed that they are biased toward T helper (Th2) type differentiation and they display an activated phenotype and enhanced proliferation [72]. In addition, production of the Th2 cytokines IL-4 and IL-5 was augmented in *itch*$^{-/-}$ T cells upon TCR stimulation alone [72]. Further evidence of Itch controlling Th2 immune responses was established in an airway inflammation model, where systemic injection of high dose of soluble antigen failed to induce tolerance in Itch-deficient mice [77]. In this model Itch has recently been implicated in negatively controlling NF-κB activation by interacting with the ubiquitin-editing enzyme A20. Proper A20 activity requires IKK activation of A20 and a complex consisting of TAXBP1/Itch and A20 [78]. The RING-type ubiquitin E3 ligase GRAIL is a type I transmembrane protein which localizes to the endocytic pathway. In response to antigen stimulation without co-stimulation GRAIL mRNA was reported to be upregulated and TCR activation of GRAIL-transfected T cell hybridomas resulted in decreased IL-2 production [71]. Retroviral transduction of hematopoietic stem cells with a dominant negative form of GRAIL and subsequent reconstitution of syngenic hosts with these cells revealed that anergy induction was blocked in naïve CD4$^+$ T cells in an *in vivo* tolerance model while over-expression of GRAIL using the same system rendered naïve CD4$^+$ T cells anergic [79]. Finally, evidence from multiple laboratories has implicated Cbl-b as a key gene involved in the induction of T cell anergy *in vitro* and *in vivo* [1,2,62,80–82].

5.5 Molecular function and regulation of Cbl-b

Cbl-b is a member of the highly conserved family of Cbl proteins. The nomenclature derives from the discovery of the murine Cas NS-1 retroviral oncoprotein v-Cbl, which promotes B-lineage lymphoma development in mice; v-Cbl encodes a dominant mutant antagonizing the function of its cellular homologue c-Cbl [83,84]. Structural analysis revealed that Cbl-family proteins function as E3 ubiquitin ligases and molecular adaptors (Figure 5.2). The mammalian family consists of three functional *cbl* genes: *c-cbl*, *cbl-b* and *cbl-3* (also called *cbl-c*) [83,85–87]. Expression profiling revealed that c-Cbl and Cbl-b are expressed in a wide range of tissues while Cbl-3 expression appears to be restricted to epithelia [84–89].

In lymphocytes, both c-Cbl and Cbl-b can control antigen-receptor signalling by ubiquitination of activated receptors, receptor-associated tyrosine kinases, as well as downstream signalling molecules to fine tune antigen-receptor signalling and immune responses [90]. c-Cbl and Cbl-b have a common domain structure and are reported to have multiple overlapping targets; however, their display differences in the level and positions of phosphorylated residues, which may explain their different functions. Whereas c-Cbl is highly phosphorylated upon TCR stimulation, which is a functional requirement for its interaction with Vav1 or CrkL, antigen-receptor-induced tyrosine

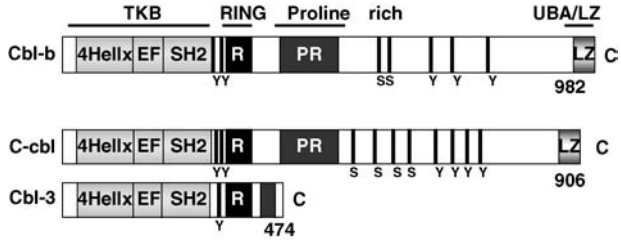

Figure 5.2 The domain structure of the Cbl family proteins of ubiquitin E3 ligases. Domain architecture of the three mammalian Cbl isoforms (c-Cbl, Cbl-b, Cbl-3) with amino acid numbers of human Cbl proteins are shown. The N-terminal tyrosine-kinase-binding (TKB) region consists of a four-helix bundle, an EF hand and an SH2 domain, and is required for target binding. This domain is connected via a linker to the RING finger (except v-Cbl, which is missing the RING finger), which confers E3 ligase activity by binding the E2 enzyme. In the C-terminal region proline-rich stretches (PR), multiple serine (S) and tyrosine (Y) phosporylation sites for protein–protein interaction, and a leucine zipper (LZ) are indicated. The LZ domain is also referred to as a ubiquitin-association domain (UBA) required to form homo- or heterodimers.

phosphorylation of Cbl-b is negligible [91]. Differential phosphorylation of c-Cbl and Cbl-b also directs interactions towards the same target; for instance, the phosphoinositide-3-kinase (PI3K) subunit p85 [92–95]. Although multiple binding partners have been reported for c-Cbl, the genetic data *in vivo* suggest that c-Cbl might primarily function in TCR downmodulation and the regulation of Zap70 in thymocytes [96]. Further, c-Cbl and Cbl-b display distinct expression patterns in T cell subtypes. Whereas c-Cbl expression is high in thymocytes compared to Cbl-b, the highest levels of Cbl-b expression can be found in peripheral T cells, suggesting a prominent role of c-Cbl in thymocytes, while Cbl-b may function as a main regulator in peripheral lymphocytes [90]. However, this of course does not exclude a potential cooperative or compensatory function of c-Cbl and Cbl-b in immune cells or other tissues. For instance, both Cbl proteins in concert appear to control thymocyte maturation at the pre-TCR stage. Therefore *c-cbl* and *cbl-b* double deficiency results in establishment of a distinct and MHC-independent $CD4^+$ and $CD8^+$ T cell repertoire [90,97].

A key target of Cbl-b is the p85 subunit of PI3K. Ubiquitylation of p85 by Cbl-b in Jurkat cells does not result in p85 degradation but influences the subcellular localization and interferes with the association of p85 with the cytoplasmic regions of CD28 and TCRζ [93]. Another important target for Cbl-b in T cells that has also been studied extensively in multiple genetic models is Vav1. Again, Cbl-b does not seem to target Vav1 for degradation, but Cbl-b controls Vav1 phosphorylation downstream of TCR signalling and CD28 co-stimulation [98]. Importantly, antigen-receptor engagement alone is sufficient to activate Vav1 and subsequently immune-synapse formation in *cbl-b*$^{-/-}$ T cells, whereas in wild-type T cells TCR plus CD28 co-stimulation are required for Vav1 activation [98]. Cbl-b has further been shown to negatively regulate

CrkL-/C3G-mediated clustering of adhesion receptors and *cbl-b*-deficient T cell lines display enhanced formation of the CrkL–C3G complex as well as Rap1-induced LFA1 clustering [99]. Loss of Cbl-b in anergic T cells has been shown to alter ubiquitination of phospholipase C (PLC) γ1, resulting in changed calcium signalling [2], suggesting that PLCγ1 might represent another key target for Cbl-b in T cells. Further, Cbl-b constitutively binds Grb2 and Zap70; however, the *in vivo* relevance of these interactions are not yet known [99]. A recent study suggests a potential negative role of Cbl-b in NF-κB activation upon Toll-like receptor (TLR)-4 stimulation. Cbl-b appears to interact directly with TLR-4 and regulate its expression as well as association with the important signalling adaptor Myd88 in response to lipopolysaccharide (LPS) stimulation. Interestingly, this function requires a functional RING domain and does not lead to proteasomal degradation of TLR-4 [100].

Since Cbl-b targets central mediators of TCR-mediated signalling and genetically is a critical regulator of T cell activation and anergy induction, its expression and function have to be tightly regulated. Cbl-b expression and degradation have been shown to be regulated by the central co-stimulatory surface receptor CD28 and the inhibitory receptor cytotoxic T lymphocyte antigen 4 (CTLA-4). As mentioned above stimulation via CD28 is essential for naïve T cell priming, inducing activation, proliferation, survival and cytokine production and thereby preventing T cell anergy [30,101,102]. However, this second co-stimulatory trigger appears to be less relevant for the reactivation of effector and memory T cells. CTLA-4 expression is transiently unregulated upon activation of naïve T cells. In contrast Tregs and anergic T cells constitutively express CTLA-4 on the cell surface [103–105]. CTLA-4 is believed to mediate its inhibitory effect via binding to CD28 ligands, therefore out-competing binding of CD28 and preventing or terminating CD28 co-stimulation. In addition, CTLA-4 has been reported to exhibit additional, CD28-independent, inhibitory effects on IL-2 production and proliferation [106] and CTLA-4 might be required for the induction of a 'deep' anergic state, which cannot be reversed by exogenous IL-2 [107–113].

Interestingly both CD28 and CTLA-4 receptors differentially regulate Cbl-b. While CD28 engagement to B7 ligands has been shown to induce Cbl-b ubiquitination and Cbl-b degradation [114–116], CTLA-4 engagement leads to upregulation of Cbl-b accompanied by suppression of T cell proliferation. A recent study underlines the relevance of Cbl-b regulation by showing that in the absence of Cbl-b CD28 engagement does not enhance T cell activation and nor does CTLA-4 engagement block proliferation [116]. However, one has to keep in mind that Cbl-b deficiency cannot fully rescue the lack of CD28 [62–64], suggesting that CD28 must act in other pathways independent of Cbl-b. Nonetheless, taking into account the importance of CD28 and CTLA-4, regulation of Cbl-b expression appears to be one key pathway by which these receptors control T cell activation.

In the ionomycin-induced tolerance model Ca^{2+}-mediated signalling results in NFAT activation without concomitant AP-1 or NF-κB activation, which results in cell-cycle arrest and initiates a suppressive transcriptional programme [10,60,61], including upregulation of Cbl-b expression as well as other E3 ligases involved in tolerance induction [1]. Under these tolerogenic conditions Cbl-b expression appears

to be mediated by the zinc-finger transcription factors Egr-2 and Egr-3 (early growth response) [82]. Recently it has been reported that the essential Treg transcription factor FoxP3 regulates Cbl-b expression via Egrs to establish anergy or conversion of effector T cells [80]. Another study observed the failure of Treg induction and failure of FoxP3 upregulation of *cbl-b*$^{-/-}$ T cells in response to TGF-β stimulation *in vitro*, suggesting that Cbl-b as a negative regulator of Smad2 phosphorylation [81]. Therefore the exact molecular interplay between FoxP3 and Cbl-b and mechanisms of TGF-β-mediated Cbl-b regulation need to be further established and might depend on the experimental system. Interestingly, Itch and GRAIL have also been shown to be upregulated by FoxP3 [80].

Recently β-catenin has been linked to anergy induction and Cbl-b and other E3 ligases. Stable expression of a β-catenin construct in naïve CD4$^+$CD25$^-$ cells resulted in an anergic T cell phenotype accompanied by upregulation of Cbl-b as well as GRAIL and Itch [117]. Besides transcriptional regulation, Cbl-b expression is also controlled by post-translational modification like ubiquitination. Cbl-b has been reported to undergo auto-ubiquitination-mediated degradation upon TCR and CD28 co-stimulation [114]. Further, Cbl-b can interact and be a target of the HECT family E3 ubiquitin ligases Itch and NEDD4 [118].

5.6 Physiological relevance of Cbl-b

Genetic proof of the *in vivo* function of Cbl-b and of the other Cbl family members comes from gene-targeting experiments in mice. Cbl-knockout mice are all viable and display no overt defects in development [62,63,89,96,97]. However *c-cbl*$^{-/-}$ and *cbl-b*$^{-/-}$ double deficiencies are embryonically lethal in mice before mid-gestation [90], suggesting molecular cooperation of c-Cbl and Cbl-b proteins in development. Whereas the *in vivo* function of Cbl-3 remains elusive [89] the important role of *c-cbl* and *cbl-b* in the immune system was demonstrated in *c-cbl*- and *cbl-b*-deficient mice [62,63,96,97].

Loss of *c-cbl* has been shown to affect thymocyte maturation primarily, resulting in elevated thymocyte numbers, elevated expression levels of CD3, TCR, CD4, CD5 and CD69, delayed activation-induced TCR downmodulation and Zap70 and MAPK hyperactivation following antigen-receptor stimulation, whereas activation of PI3K and PLCγ1 activation were paradoxically reduced [119]. Positive selection for mature CD4$^+$ cells was enhanced, while positive selection of CD8$^+$ lineage T cells was unchanged and *c-cbl*$^{-/-}$ mice exhibited increased splenic weight as well as impaired TCR stimulation in peripheral tissue [96,119].

Genetic experiments performed in our laboratory and others have demonstrated that Cbl-b plays a major role as a negative regulator of peripheral lymphocyte activation. *Cbl-b*$^{-/-}$ mice do not display apparent alterations in thymic selection as compared with *c-cbl*-deficient mice as assessed by T cell development in a polyclonal system as well as TCR transgenic models [62,63]. Instead, peripheral T cells lacking Cbl-b are hyper-responsive to TCR stimulation and loss of Cbl-b results in increased

T cell proliferation and IL-2 production even in the absence of CD28 co-stimulation. This shows that Cbl-b can uncouple T cell activation from the requirement for CD28 co-stimulation [62,63]. Indeed, Cbl-b deficiency has been shown to rescue some of the functional defects observed in *cd28*$^{-/-}$ T cells [64,120]; antigen receptor stimulation alone appears to be sufficient to induce strong Vav1 activation and receptor clustering, providing a molecular explanation of how Cbl-b controls antigen-receptor stimulation and autoimmunity. Further genetic deletion of *cbl-b* and *vav1* confirmed that Cbl-b regulates calcium flux and IL-2 production through Vav1 [65]. As a rational consequence of T cell hyper-responsivness *cbl-b*$^{-/-}$ mice display enhanced sensitivity to spontaneous and peptide-induced autoimmunity. Cbl-b has further been implicated in the regulation of activation-induced cell death in CD4$^+$ T cells: whereas wild-type Th1 cells undergo apoptosis in response to CD3 stimulation in the absence of CD28 co-stimulation, *cbl-b*$^{-/-}$ Th1 cells appear to be resistant to activation-induced cell death [121].

Recently, the functional consequence of a combined loss of c-Cbl and Cbl-b in thymic development as well as in the periphery has been addressed using *c-cbl* conditional floxed alleles together with a Cre deleter line specific for the lymphocyte lineage crossed into a *cbl-b* mutant background [90,97]. In the thymus, double-deficient mice displayed reduced numbers of double-positive thymocytes, possibly as a result of enhanced negative selection of both CD4$^+$ and CD8$^+$ T cell lineage cells. Surprisingly, simultaneous ablation of c-Cbl and Cbl-b in thymocytes led to the development of mature CD4$^+$ and CD8$^+$ T cells even in the absence of MHC class I or II expression. Pre-TCR failed to be downregulated in CD4$^+$CD8$^+$ thymocytes of *c-cbl*$^{-/-}$*cbl-b*$^{-/-}$ double-deficient mice and the resulting continuous pre-TCR stimulation might explain enhanced NF-κB activation independent of MHC [97]. In the periphery, these *c-cbl*$^{-/-}$*cbl-b*$^{-/-}$ double-deficient mice develop severe autoimmune organ infiltration, splenomegaly and autoantibodies, and die between 12 and 16 weeks of age [90]. T cells display an effector-memory phenotype and show even higher proliferation compared to *cbl-b* single-deficient cells in response to anti-CD3 stimulation. However, further biochemical as well as *in vivo* experiments are required to dissect the cross-talk of these two Cbl proteins with each other and with other pathways operational in development, differentiation and activation of T cells.

5.7 The role of Cbl-b in T cell tolerance

A key finding was that genetic ablation of *cbl-b* resulted in high susceptibility to spontaneous and induced experimental autoimmune diseases [2,62,63,122]. *cbl-b* deficiency *in vivo* results in the development of spontaneous autoimmune organ infiltration starting between 3 and 6 months of age [62]. In an independently derived *cbl-b*$^{-/-}$ mouse strain, immunization with myelin basic protein triggered dramatically increased experimental autoimmune encephalomyelitis [63]. We could

further establish that *cbl-b*$^{-/-}$ mice develop collagen-induced arthritis even in the absence of microbacterial adjuvant stimulation [2]. The ultimate evidence for the essential role of Cbl-b in peripheral T cell tolerance comes from *in vivo* antigen-specific tolerance experiments. Intriguingly, whereas repeated exposure of P14 TCR transgenic mice to the cognate p33 antigen resulted in the induction of T cell anergy, repeated challenge of P14 TCR transgenic *cbl-b*$^{-/-}$ mice led to massive activation of CD8$^+$ T cells and in many cases death of the mice due to a T cell hyperactivation and cytokine storm [2]. Moreover, tolerance induction was impaired in CD4$^+$ *cbl-b*$^{-/-}$ T cells expressing the ovalbumin-reactive TCR in a transfer model system. Finally, repeated injections of the superantigen staphylococcal enterotoxin B (SEB) also did not result in tolerance induction; instead, *cbl-b*$^{-/-}$ mice displayed elevated cytokine levels in the plasma. Thus, the presence of Cbl-b can determine the fate of mice to survive or die in response to repeated antigen exposure.

In a model for virally induced autoimmune diabetes, the transgenic P14/Rip-gp model, an additional role of Cbl-b in establishing the activation threshold became apparent. While lymphocytic choriomeningitis virus (LCMV) infection results in development of diabetes in P14 transgenic mice the low agonistic peptide variant LCMV-LF6 triggers diabetes only in less than 50% of the infected mice. However, infection of P14 TCR transgenic *cbl-b*$^{-/-}$ mice with LCMV-LF6 led to diabetes development in all mice with rapid disease progression, enhanced T cell proliferation and increased cytotoxic T lymphocyte (CTL) effector function [122]. Thus, Cbl-b was the first E3 ligase ever found that was directly linked to T cell activation and T cell tolerance *in vivo*. In addition to impaired tolerance induction *in vivo*, *cbl-b*-deficient T cells have shown resistance to anergy induction in response to ionomycin, TCR stimulation in the absence of CD28 co-stimulation or exposure to TGF-β [1,81]. Cbl-b has recently been described to enhance inflammation and morbidity in an LPS-induced model of acute lung injury by increased sepsis-induced release of cytokines. This effect appears to depend on Cbl-b regulating TLR-4-mediated NF-κB activation [100].

In addition to intrinsic control of T cell anergy, Cbl-b appears to have a role in Treg-mediated suppression. Natural CD25$^+$CD4$^+$ Tregs occur at comparable numbers in *cbl-b*-deficient mice and do not show obvious impairment in their development or function *in vitro* [81,123]. However, *cbl-b*$^{-/-}$ CD4$^+$ [81] as well as *cbl-b*$^{-/-}$ CD8$^+$ [123] T cells show reduced sensitivity to Treg-mediated suppression in classical *in vitro* suppression assays. However, when Tregs are co-cultured with CD4$^+$ or CD8$^+$ *cbl-b*$^{-/-}$ effector cells at high Treg/T effector cell ratios, suppression can be observed although it is reduced compared to wild-type effector cells [81,124]. Moreover, *cbl-b*-deficient T cells fail to upregulate FoxP3 *in vitro* in response to antigen stimulation in the presence of TGF-β and are therefore resistant to Treg conversion [124]. Furthermore, *cbl-b*-deficient effector CD4$^+$ T cells have been shown to be resistant to TGF-β-mediated suppression [81,124]. However *cbl-b* deficiency does not phenocopy Treg-deficient or TGF-β-deficient mice and therefore Cbl-b functions can certainly not be explained only by resistance to Treg suppression.

Interestingly Itch-deficient T cells also display resistance to Treg suppression *in vitro* and fail to upregulate FoxP3 in the TGF-β conversion model [77].

5.8 Deregulation of Cbl-b in disease

A role of Cbl-b has not only been described in mouse models, but Cbl-b has also been found to be dysregulated in autoimmunity in other species. For instance, Cbl-b has been found to be truncated and is therefore a major susceptibility gene for spontaneous type 1 diabetes development in the Komeda diabetes-prone (KDP) rat. Intriguingly, complementation of the Cbl-b mutation in transgenic rescue experiments with wild-type *cbl-b* suppressed the development of type 1 diabetes in a gene-dosage-dependent manner, confirming that Cbl-b is a key type 1 diabetes susceptibility gene in rodents [125,126].

Due to the high conservation among species, attempts have been made to also screen for alterations in Cbl-b in human diabetes. A recent human study [127] linked type 1 diabetes with an exon-12 polymorphism encoding the proline-rich domain of Cbl-b. A genetic interaction between this Cbl-b single nucleotide polymorphism (SNP), the CTLA-4 SNP C60 and susceptibility to type 1 diabetes was further suggested. Interestingly, the same study identified a splice variant of Cbl-b lacking exon 16 in peripheral blood mononuclear cells which was specifically upregulated following cytokine stimulation. However, expression of this Cbl-b isoform did not correlate with susceptibility to diabetes [127].

Cbl-b has been further found to be upregulated during chronic infections in humans. For instance, it has been reported that Cbl-b is upregulated in peripheral blood mononuclear cells from HIV patients [128]. Whether Cbl-b directly accounts for defective proximal signalling and impaired ERK activation leading to unresponsiveness of HIV-infected $CD4^+$ and $CD8^+$ T cells needs to be determined. Cbl-b has also been found to be upregulated in patients with chronic helminth infections or lymphatic filariasis. Increased Cbl-b levels through CTLA-4 upregulation and TGF-β stimulation could possibly explain T cell hyporesponsiveness in helminth infections [129] or filaria-induced immune suppression [130]. A recent publication reported a novel role of Cbl-b in pathogen elimination. Cbl-b appears to target the key virulence determinant Exotoxin T (ExoT) of *Pseudomonas aeroginosa* for degradation, thereby protecting against dissemination of this bacterium [131]. This opportunistic human pathogen is a leading cause of nosocomial infections.

5.9 Therapeutic potential of Cbl-b in tumour immunity

Genetic inactivation of Cbl-b has a dramatic impact on T cell activation and results in impaired T cell anergy. Therefore, reduction of Cbl-b expression or inhibition of its function might allow us to interfere with a T cell-unresponsive state as seen in HIV or cancer patients. Our group [123] and others [132] recently explored the role of Cbl-b in

immune-mediated tumour rejection. Intriguingly, *cbl-b*-deficient mice displayed spontaneous tumour rejection in various tumour models of solid as well as haematopoietic cancer [123,132]. Using the TC-1 cancer model, a model based on cancer development by subcutaneous injection of the human papilloma virus (HPV)- and cHras-transformed C57BL/6 lung fibroblast cell line TC-1, $CD8^+$ T cells have been identified as critical players in the rejection process, but not $CD4^+$ T cells [123]. Chiang and colleagues showed, with a different *cbl-b*-deficient mouse strain [132], that these mice also reject highly immunogenic EG7 cells, an EL-4-derived cell line expressing ovalbumin as an model antigen, or even EL-4 cells not expressing the antigen and considered of low immunogenicity.

Importantly, these findings could also be recapitulated in a spontaneous tumour model. *cbl-b*$^{-/-}$ mice developed dramatically reduced skin tumours in an ultraviolet B-induced spontaneous skin cancer model compared to the wild-type cohort. In addition, tumours developed in *cbl-b*$^{-/-}$ mice were reduced in their size after initial progressive growth. However, depletion of $CD8^+$ cells in ultraviolet B-treated tumour-free *cbl-b*$^{-/-}$ mice results in rapid and progressive tumour outgrowth [123]. In a different spontaneous tumour model using ataxia telangiectasia mutated (ATM)-deficient mice, which spontaneously develop T cell lymphomas, Cbl-b deficiency could also reduce morbidity in those mice [132].

The relevance of $CD8^+$ CTLs and their therapeutic potential was subsequently tested in a therapeutic transfer system. Rag-2$^{-/-}$ mice, which lack T cells, were injected with TC-1 tumour cells followed by a transfer of naïve $CD8^+$ cells. Whereas transfer of naïve *cbl-b*$^{-/-}$ $CD8^+$ T cells was sufficient to dramatically reduce tumour growth, mice injected with wild-type $CD8^+$ T cells displayed progressive tumour growth [123]. Additionally, adoptive transfer of $CD8^+$ T cells into mice bearing EG7-induced tumours reduced tumour burden. Again, $CD8^+$ T cells were identified by depletion, genetic deletion and experimental transfer as the essential mediators of tumour rejection in *cbl-b*$^{-/-}$ mice.

Mechanistically, deletion of *cbl-b* might affect anti-cancer immunity at several levels. For efficient control of tumour growth it is important that the immune system becomes alert to the growing tumour at early stages. We found that *cbl-b*-deficient $CD8^+$ T cells infiltrate the tumour earlier and are more abundant in the tumour tissue. Further analysis of the tumour-draining lymph nodes revealed elevated numbers of tumour-specific CD8 cells displaying effector functions as determined by interferon-γ production. While Treg function appears normal in *cbl-b*$^{-/-}$ mice, $CD8^+$ effector cells show partial resistance to Treg-mediated suppression. We could also observe efficient tumour rejection upon rechallenge of *cbl-b*-deficient mice with a 100-times lethal dose of TC-1 cells 1 year after first challenge, suggesting efficient and long-lasting tumour memory in *cbl-b*$^{-/-}$ mice [123]. However, the detailed mechanism of tumour-specific activation and killing in *cbl-b* deficiency still has to be determined experimentally. Nevertheless, this suggests that inactivation of a single central negative regulator Cbl-b, is sufficient to convey spontaneous tumour rejection and protection by CTLs in different tumour models. Therefore Cbl-b is a new, very promising target for cancer immunotherapy.

5.10 Implications for autoimmune disease

The proper balance of T cell activation with T cell tolerance is crucial for protective immunity while preventing autoimmunity. A key molecule in this process is the E3 ligase Cbl-b. Cbl-b has been shown to control the threshold of TCR activation and CD28 co-stimulation by interfering with a number of essential signalling molecules upon antigen-specific stimulation. Genetic inactivation of this negative player has a dramatic impact on T cell activation and proliferation capacities, and results in increased autoimmunity and impaired T cell anergy. In addition, Cbl-b appears to be involved in Treg conversion and Treg suppression of effector T cells. Genetic ablation of *cbl-b* resulted in high susceptibility to spontaneous and induced experimental autoimmune diseases [2,62,63,122]. In mice, but also in rats, truncation in *cbl-b* has been found to be a major susceptibility gene for spontaneous type 1 diabetes development [125,126]. More recently a human study also linked an SNP in the *cbl-b* gene to human diabetes susceptibility [127]; however, other earlier studies in human diabetes [133] as well as Gave's disease could not link polymorphisms in the *cbl-b* gene with disease susceptibility [134]. Therefore the role of Cbl-b in susceptibility to autoimmune disease requires further analysis. However, since high levels of Cbl-b can prevent autoimmunity in diabetic rats, restoring or enhancing Cbl-b expression/function might be a useful tool in preventing autoimmunity or in inducing specific tolerance in organ transplantation. On the other hand, reduction or inhibition of Cbl-b might allow us to interfere with a T cell-unresponsive state, as seen in HIV infection or cancer. Indeed, initial studies now focus on Cbl-b as a promising new target for tumour immunotherapy. In conclusion, Cbl-b modulation might be a fascinating novel modality with which to control immunity in vaccination, cancer biology, chronic infections or autoimmunity such as type 1 diabetes.

References

1. Heissmeyer, V., Macian, F., Im, S.H. *et al.* (2004) Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins. *Nature Immunology* **5**, 255–265.
2. Jeon, M.S., Atfield, A., Venuprasad, K. *et al.* (2004) Essential role of the E3 ubiquitin ligase Cbl-b in T cell anergy induction. *Immunity* **21**, 167–177.
3. Goodnow, C.C., Sprent, J., Fazekas de St Groth, B. *et al.* (2005) Cellular and genetic mechanisms of self tolerance and autoimmunity. *Nature* **435**, 590–597.
4. Venanzi, E.S., Benoist, C., Mathis, D. (2004) Good riddance: Thymocyte clonal deletion prevents autoimmunity. *Current Opinion in Immunology* **16**, 197–202.
5. Mathis, D. and Benoist, C. (2004) Back to central tolerance. *Immunity* **20**, 509–516.
6. Kyewski, B. and Derbinski, J. (2004) Self-representation in the thymus: an extended view. *Nature Reviews Immunology* **4**, 688–698.
7. Filion, M.C., Bradley, A.J., Devine, D.V. *et al.* (1995) Autoreactive T cells in healthy individuals show tolerance *in vitro* with characteristics similar to but distinct from clonal anergy. *European Journal of Immunology* **25**, 3123–3127.
8. Fontenot, J.D. and Rudensky, A.Y. (2005) A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nature Immunology* **6**, 331–337.

9. Sakaguchi, S., Ono, M., Setoguchi, R. *et al.* (2006) Foxp3 + CD25 + CD4 + natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunological Reviews* **212**, 8–27.
10. Borde, M., Barrington, R.A., Heissmeyer, V. *et al.* (2006) Transcriptional basis of lymphocyte tolerance. *Immunological Reviews* **210**, 105–119.
11. Appleman, L.J. and Boussiotis, V.A. (2003) T cell anergy and costimulation. *Immunological Reviews* **192**, 161–180.
12. Bluestone, J.A. and Abbas, A.K. (2003) Natural versus adaptive regulatory T cells. *Nature Reviews Immunology* **3**, 253–257.
13. Hori, S., Nomura, T. and Sakaguchi, S. (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057–1061.
14. Fontenot, J.D., Gavin, M.A. and Rudensky, A.Y. (2003) Foxp3 programs the development and function of CD4 + CD25 + regulatory T cells. *Nature Immunology* **4**, 330–336.
15. Sakaguchi, S., Sakaguchi, N., Asano, M. *et al.* (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *Journal of Immunology* **155**, 1151–1164.
16. Herbelin, A., Gombert, J.M., Lepault, F. *et al.* (1998) Mature mainstream TCR alpha beta + CD4 + thymocytes expressing L-selectin mediate "active tolerance" in the nonobese diabetic mouse. *Journal of Immunology* **161**, 2620–2628.
17. Hall, B.M., Pearce, N.W., Gurley, K.E. *et al.* (1990) Specific unresponsiveness in rats with prolonged cardiac allograft survival after treatment with cyclosporine. III. Further characterization of the CD4 + suppressor cell and its mechanisms of action. *Journal of Experimental Medicine* **171**, 141–157.
18. Gambineri, E., Torgerson, T.R. and Ochs, H.D. (2003) Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), a syndrome of systemic autoimmunity caused by mutations of FOXP3, a critical regulator of T-cell homeostasis. *Current Opinion in Rheumatology* **15**, 430–435.
19. Roncarolo, M.G., Gregori, S., Battaglia, M. *et al.* (2006) Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunological Reviews* **212**, 28–50.
20. Faria, A.M. and Weiner, H.L. (2005) Oral tolerance. *Immunological Reviews* **206**, 232–259.
21. Chang, C.C., Ciubotariu, R., Manavalan, J.S. *et al.* (2002) Tolerization of dendritic cells by T(S) cells: the crucial role of inhibitory receptors ILT3 and ILT4. *Nature Immunology* **3**, 237–243.
22. Kronenberg, M. (2005) Toward an understanding of NKT cell biology: progress and paradoxes. *Annual Review of Immunology* **23**, 877–900.
23. Zhang, Z.X., Yang, L., Young, K.J. *et al.* (2000) Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. *Nature Medicine* **6**, 782–789.
24. Hayday, A. and Tigelaar, R. (2003) Immunoregulation in the tissues by gammadelta T cells. *Nature Reviews Immunology* **3**, 233–242.
25. Tang, Q. and Bluestone, J.A. (2008) The Foxp3 + regulatory T cell: a jack of all trades, master of regulation. *Nature Immunology* **9**, 239–244.
26. Schwartz, R.H. (2003) T cell anergy. *Annual Review of Immunology* **21**, 305–334.
27. Jenkins, M.K. and Schwartz, R.H. (1987) Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness *in vitro* and *in vivo*. *Journal of Experimental Medicine* **165**, 302–319.
28. Schwartz, R.H., Mueller, D.L., Jenkins, M.K. *et al.* (1989) T-cell clonal anergy *Cold Spring Harbor Symposia on Quantitative Biology* **54**(2), 605–610.
29. Salomon, B. and Bluestone, J.A. (2001) Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. *Annual Review of Immunology* **19**, 225–252.
30. Harding, F.A., McArthur, J.G., Gross, J.A. *et al.* (1992) CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature* **356**, 607–609.
31. Jenkins, M.K., Taylor, P.S., Norton, S.D. *et al.* (1991) CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *Journal of Immunology* **147**, 2461–2466.

32. Tuosto, L. and Acuto, O. (1998) CD28 affects the earliest signaling events generated by TCR engagement. *European Journal of Immunology* **28**, 2131–2142.
33. Burr, J.S., Savage, N.D., Messah, G.E. *et al.* (2001) Cutting edge: distinct motifs within CD28 regulate T cell proliferation and induction of Bcl-XL. *Journal of Immunology* **166**, 5331–5335.
34. Okkenhaug, K., Wu, L., Garza, K.M. *et al.* (2001) A point mutation in CD28 distinguishes proliferative signals from survival signals. *Nature Immunology* **2**, 325–332.
35. Bluestone, J.A. (1995) New perspectives of CD28-B7-mediated T cell costimulation. *Immunity* **2**, 555–559.
36. Viola, A., Schroeder, S., Sakakibara, Y. *et al.* (1999) T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science* **283**, 680–682.
37. Sperling, A.I., Auger, J.A., Ehst, B.D. *et al.* (1996) CD28/B7 interactions deliver a unique signal to naive T cells that regulates cell survival but not early proliferation. *Journal of Immunology* **157**, 3909–3917.
38. Fraser, J.D., Irving, B.A., Crabtree, G.R. *et al.* (1991) Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science* **251**, 313–316.
39. Thompson, C.B., Lindsten, T., Ledbetter, J.A. *et al.* (1989) CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. *Proceedings of the National Academy of Sciences, USA* **86**, 1333–1337.
40. Norton, S.D., Zuckerman, L., Urdahl, K.B. *et al.* (1992) The CD28 ligand, B7, enhances IL-2 production by providing a costimulatory signal to T cells. *Journal of Immunology* **149**, 1556–1561.
41. Curtsinger, J.M., Schmidt, C. S., Mondino, A. *et al.* (1999) Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells. *Journal of Immunology* **162**, 3256–3262.
42. Curtsinger, J.M., Lins, D.C., Johnson, C.M. *et al.* (2005) Signal 3 tolerant CD8 T cells degranulate in response to antigen but lack granzyme B to mediate cytolysis. *Journal of Immunology* **175**, 4392–4399.
43. Lechler, R., Chai, J.G., Marelli-Berg, F. *et al.* (2001) The contributions of T-cell anergy to peripheral T-cell tolerance. *Immunology* **103**, 262–269.
44. Appleman, L.J., Tzachanis, D., Grader-Beck, T. *et al.* (2001) Helper T cell anergy: from biochemistry to cancer pathophysiology and therapeutics. *Journal of Molecular Medicine* **78**, 673–683.
45. Mirshahidi, S., Huang, C.T. and Sadegh-Nasseri, S. (2001) Anergy in peripheral memory CD4 (+) T cells induced by low avidity engagement of T cell receptor. *Journal of Experimental Medicine* **194**, 719–731.
46. Choi, S. and Schwartz, R.H. (2007) Molecular mechanisms for adaptive tolerance and other T cell anergy models. *Seminars in Immunology* **19**, 140–152.
47. Quill, H., Riley, M.P., Cho, E.A. *et al.* (1992) Anergic Th1 cells express altered levels of the protein tyrosine kinases p56lck and p59fyn. *Journal of Immunology* **149**, 2887–2893.
48. Fields, P.E., Gajewski, T.F. and Fitch, F.W. (1996) Blocked Ras activation in anergic CD4+ T cells. *Science* **271**, 1276–1278.
49. Kang, S.M., Beverly, B., Tran, A.C. *et al.* (1992) Transactivation by AP-1 is a molecular target of T cell clonal anergy. *Science* **257**, 1134–1138.
50. Li, W., Whaley, C.D., Mondino, A. *et al.* (1996) Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4+ T cells. *Science* **271**, 1272–1276.
51. Rudd, C.E. (2006) Cell cycle 'check points' T cell anergy. *Nature Immunology* **7**, 1130–1132.
52. Zha, Y., Marks, R., Ho, A.W. *et al.* (2006) T cell anergy is reversed by active Ras and is regulated by diacylglycerol kinase-alpha. *Nature Immunology* **7**, 1166–1173.
53. Olenchock, B.A., Guo, R., Carpenter, J.H. *et al.* (2006) Disruption of diacylglycerol metabolism impairs the induction of T cell anergy. *Nature Immunology* **7**, 1174–1181.
54. Zhong, X.P., Hainey, E.A., Olenchock, B.A. *et al.* (2003) Enhanced T cell responses due to diacylglycerol kinase zeta deficiency. *Nature Immunology* **4**, 882–890.
55. Crespi, D., Massa, S., Basso, V. *et al.* (2002) Constitutive active p21ras enhances primary T cell responsiveness to Ca2+ signals without interfering with the induction of clonal anergy. *European Journal of Immunology* **32**, 2500–2509.

56. Mueller, D.L. (2006) Linking diacylglycerol kinase to T cell anergy. *Nature Immunology* **7**, 1132–1134.
57. Rowell, E.A., Walsh, M.C. and Wells, A.D. (2005) Opposing roles for the cyclin-dependent kinase inhibitor p27kip1 in the control of CD4+ T cell proliferation and effector function. *Journal of Immunology* **174**, 3359–3368.
58. Boussiotis, V.A., Freeman, G.J., Taylor, P.A. *et al.* (2000) p27kip1 functions as an anergy factor inhibiting interleukin 2 transcription and clonal expansion of alloreactive human and mouse helper T lymphocytes. *Nature Medicine* **6**, 290–297.
59. Li, L., Iwamoto, Y., Berezovskaya, A. *et al.* (2006) A pathway regulated by cell cycle inhibitor p27Kip1 and checkpoint inhibitor Smad3 is involved in the induction of T cell tolerance. *Nature Immunology* **7**, 1157–1165.
60. Macian, F., Garcia-Cozar, F., Im, S.H. *et al.* (2002) Transcriptional mechanisms underlying lymphocyte tolerance. *Cell* **109**, 719–731.
61. Macian, F. (2005) NFAT proteins: key regulators of T-cell development and function. *Nature Reviews Immunology* **5**, 472–484.
62. Bachmaier, K., Krawczyk, C., Kozieradzki, I. *et al.* (2000) Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature* **403**, 211–216.
63. Chiang, Y.J., Kole, H.K., Brown, K. *et al.* (2000) Cbl-b regulates the CD28 dependence of T-cell activation. *Nature* **403**, 216–220.
64. Krawczyk, C.M., Jones, R.G., Atfield, A. *et al.* (2005) Differential control of CD28-regulated *in vivo* immunity by the E3 ligase Cbl-b. *Journal of Immunology* **174**, 1472–1478.
65. Krawczyk, C., Bachmaier, K., Sasaki, T. *et al.* (2000) Cbl-b is a negative regulator of receptor clustering and raft aggregation in T cells. *Immunity* **13**, 463–473.
66. Pickart, C.M. (2001) Mechanisms underlying ubiquitination. *Annual Review of Biochemistry* **70**, 503–533.
67. Weissman, A.M. (2001) Themes and variations on ubiquitylation. *Nature Reviews Molecular Cell Biology* **2**, 169–178.
68. Haglund, K., Sigismund, S., Polo, S. *et al.* (2003) Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. *Nature Cell Biology* **5**, 461–466.
69. Liu, Y.C. (2004) Ubiquitin ligases and the immune response. *Annual Review of Immunology* **22**, 81–127.
70. Soares, L., Seroogy, C., Skrenta, H. *et al.* (2004) Two isoforms of otubain 1 regulate T cell anergy via GRAIL. *Nature Immunology* **5**, 45–54.
71. Anandasabapathy, N., Ford, G.S., Bloom, D. *et al.* (2003) GRAIL: an E3 ubiquitin ligase that inhibits cytokine gene transcription is expressed in anergic CD4+ T cells. *Immunity* **18**, 535–547.
72. Fang, D., Elly, C., Gao, B. *et al.* (2002) Dysregulation of T lymphocyte function in itchy mice: a role for Itch in TH2 differentiation. *Nature Immunology* **3**, 281–287.
73. Angers, A., Ramjaun, A.R. and McPherson, P.S. (2004) The HECT domain ligase itch ubiquitinates endophilin and localizes to the trans-Golgi network and endosomal system. *Journal of Biological Chemistry* **279**, 11471–11479.
74. Fang, D. and Kerppola, T.K. (2004) Ubiquitin-mediated fluorescence complementation reveals that Jun ubiquitinated by Itch/AIP4 is localized to lysosomes. *Proceedings of the National Academy of Sciences, USA* **101**, 14782–14787.
75. Marchese, A., Raiborg, C., Santini, F. *et al.* (2003) The E3 ubiquitin ligase AIP4 mediates ubiquitination and sorting of the G protein-coupled receptor CXCR4. *Developmental Cell* **5**, 709–722.
76. Perry, W.L., Hustad, C.M., Swing, D.A. *et al.* (1998) The itchy locus encodes a novel ubiquitin protein ligase that is disrupted in a18H mice. *Nature Genetics* **18**, 143–146.
77. Venuprasad, K., Huang, H., Harada, Y. *et al.* (2008) The E3 ubiquitin ligase Itch regulates expression of transcription factor Foxp3 and airway inflammation by enhancing the function of transcription factor TIEG1. *Nature Immunology* **9**, 245–253.
78. Shembade, N., Harhaj, N.S., Parvatiyar, K. *et al.* (2008) The E3 ligase Itch negatively regulates inflammatory signaling pathways by controlling the function of the ubiquitin-editing enzyme A20. *Nature Immunology* **9**, 254–262.

79. Seroogy, C.M., Soares, L., Ranheim, E.A. *et al.* (2004) The gene related to anergy in lymphocytes, an E3 ubiquitin ligase, is necessary for anergy induction in CD4 T cells. *Journal of Immunology* **173**, 79–85.
80. Chang, X., Chen, L., Wen, J. *et al.* (2006) Foxp3 controls autoreactive T cell activation through transcriptional regulation of early growth response genes and E3 ubiquitin ligase genes, independently of thymic selection. *Clinical Immunology* **121**, 274–285.
81. Wohlfert, E.A., Callahan, M.K. and Clark, R.B. (2004) Resistance to CD4 + CD25 + regulatory T cells and TGF-beta in Cbl-b$^{-/-}$ mice. *Journal of Immunology* **173**, 1059–1065.
82. Safford, M., Collins, S., Lutz, M.A. *et al.* (2005) Egr-2 and Egr-3 are negative regulators of T cell activation. *Nature Immunology* **6**, 472–480.
83. Langdon, W.Y., Hartley, J.W., Klinken, S.P. *et al.* (1989) v-cbl, an oncogene from a dual-recombinant murine retrovirus that induces early B-lineage lymphomas. *Proceedings of the National Academy of Sciences, USA* **86**, 1168–1172.
84. Blake, T.J., Shapiro, M., Morse, H.C. 3rd. *et al.* (1991) The sequences of the human and mouse c-cbl proto-oncogenes show v-cbl was generated by a large truncation encompassing a proline-rich domain and a leucine zipper-like motif. *Oncogene* **6**, 653–657.
85. Keane, M.M., Ettenberg, S.A., Nau, M.M. *et al.* (1999) cbl-3: a new mammalian cbl family protein. *Oncogene* **18**, 3365–3375.
86. Keane, M.M., Rivero-Lezcano, O.M., Mitchell, J.A. *et al.* (1995) Cloning and characterization of cbl-b: a SH3 binding protein with homology to the c-cbl proto-oncogene. *Oncogene* **10**, 2367–2377.
87. Kim, M., Tezuka, T., Suziki, Y. *et al.* (1999) Molecular cloning and characterization of a novel cbl-family gene, cbl-c. *Gene* **239**, 145–154.
88. Nau, M.M. and Lipkowitz, S. (2003) Comparative genomic organization of the cbl genes. *Gene* **308**, 103–113.
89. Griffiths, E.K., Sanchez, O., Mill, P. *et al.* (2003) Cbl-3-deficient mice exhibit normal epithelial development. *Molecular and Cellular Biology* **23**, 7708–7718.
90. Naramura, M., Jang, I.K., Kole, H. *et al.* (2002) c-Cbl and Cbl-b regulate T cell responsiveness by promoting ligand-induced TCR down-modulation. *Nature Immunology* **3**, 1192–1199.
91. Thien, C.B. and Langdon, W.Y. (2001) Cbl: many adaptations to regulate protein tyrosine kinases. *Nature Reviews Molecular Cell Biology* **2**, 294–307.
92. Hartley, D., Meisner, H. and Corvera, S. (1995) Specific association of the beta isoform of the p85 subunit of phosphatidylinositol-3 kinase with the proto-oncogene c-cbl. *Journal of Biological Chemistry* **270**, 18260–18263.
93. Fang, D. and Liu, Y.C. (2001) Proteolysis-independent regulation of PI3K by Cbl-b-mediated ubiquitination in T cells. *Nature Immunology* **2**, 870–875.
94. Meisner, H., Conway, B.R., Hartley, D. *et al.* (1995) Interactions of Cbl with Grb2 and phosphatidylinositol 3′-kinase in activated Jurkat cells. *Molecular and Cellular Biology* **15**, 3571–3578.
95. Hunter, S., Burton, E.A., Wu, S.C. *et al.* (1999) Fyn associates with Cbl and phosphorylates tyrosine 731 in Cbl, a binding site for phosphatidylinositol 3-kinase. *Journal of Biological Chemistry* **274**, 2097–2106.
96. Murphy, M.A., Schnall, R.G., Venter, D.J. *et al.* (1998) Tissue hyperplasia and enhanced T-cell signalling via ZAP-70 in c-Cbl-deficient mice. *Molecular and Cellular Biology* **18**, 4872–4882.
97. Huang, F., Kitaura, Y., Jang, I. *et al.* (2006) Establishment of the major compatibility complex-dependent development of CD4 + and CD8 + T cells by the Cbl family proteins. *Immunity* **25**, 571–581.
98. Krawczyk, C. and Penninger, J.M. (2001) Molecular controls of antigen receptor clustering and autoimmunity. *Trends in Cell Biology* **11**, 212–220.
99. Elly, C., Witte, S., Zhang, Z. *et al.* (1999) Tyrosine phosphorylation and complex formation of Cbl-b upon T cell receptor stimulation. *Oncogene* **18**, 1147–1156.
100. Bachmaier, K., Toya, S., Gao, X. *et al.* (2007) E3 ubiquitin ligase Cblb regulates the acute inflammatory response underlying lung injury. *Nature Medicine* **13**, 920–926.
101. Lenschow, D.J., Walunas, T.L. and Bluestone, J.A. (1996) CD28/B7 system of T cell costimulation. *Annual Review of Immunology* **14**, 233–258.
102. Sansom, D.M. and Walker, L.S. (2006) The role of CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) in regulatory T-cell biology. *Immunological Reviews* **212**, 131–148.

103. Perkins, D., Wang, Z., Donovan, C. *et al.* (1996) Regulation of CTLA-4 expression during T cell activation. *Journal of Immunology* **156**, 4154–4159.
104. Takahashi, T., Tagami, T., Yamazaki, S. *et al.* (2000) Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *Journal of Experimental Medicine* **192**, 303–310.
105. Lechner, O., Lauber, J., Franzke, A. *et al.* (2001) Fingerprints of anergic T cells. *Current Biology* **11**, 587–595.
106. Chambers, C.A., Kuhns, M.S., Egen, J.G. *et al.* (2001) CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. *Annual Review of Immunology* **19**, 565–594.
107. Wells, A.D., Walsh, M.C., Bluestone, J.A. *et al.* (2001) Signaling through CD28 and CTLA-4 controls two distinct forms of T cell anergy. *Journal of Clinical Investigation* **108**, 895–903.
108. Fallarino, F., Fields, P.E. and Gajewski, T.F. (1998) B7-1 engagement of cytotoxic T lymphocyte antigen 4 inhibits T cell activation in the absence of CD28. *Journal of Experimental Medicine* **188**, 205–210.
109. Krummel, M.F. and Allison, J.P. (1996) CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *Journal of Experimental Medicine* **183**, 2533–2540.
110. Walunas, T.L., Bakker, C.Y. and Bluestone, J.A. (1996) CTLA-4 ligation blocks CD28-dependent T cell activation. *Journal of Experimental Medicine* **183**, 2541–2550.
111. Linton, P.J., Haynes, L., Klinman, N.R. *et al.* (1996) Antigen-independent changes in naive CD4 T cells with aging. *Journal of Experimental Medicine* **184**, 1891–1900.
112. Lee, K.M., Chuang, E., Griffin, M. *et al.* (1998) Molecular basis of T cell inactivation by CTLA-4. *Science* **282**, 2263–2266.
113. Perez, V.L., Van Parijs, L., Biuckians, A. *et al.* (1997) Induction of peripheral T cell tolerance *in vivo* requires CTLA-4 engagement. *Immunity* **6**, 411–417.
114. Zhang, J., Bardos, T., Li, D. *et al.* (2002) Cutting edge: regulation of T cell activation threshold by CD28 costimulation through targeting Cbl-b for ubiquitination. *Journal of Immunology* **169**, 2236–2240.
115. Mueller, D.L. (2004) E3 ubiquitin ligases as T cell anergy factors. *Nature Immunology* **5**, 883–890.
116. Li, D., Gal, I., Vermes, C. *et al.* (2004) Cutting edge: Cbl-b: one of the key molecules tuning CD28- and CTLA-4-mediated T cell costimulation. *Journal of Immunology* **173**, 7135–7139.
117. Ding, Y., Shen, S., Lino, A.C. *et al.* (2008) Beta-catenin stabilization extends regulatory T cell survival and induces anergy in nonregulatory T cells. *Nature Medicine* **14**, 162–169.
118. Magnifico, A., Ettenberg, S., Yang, C. *et al.* (2003) WW domain HECT E3s target Cbl RING finger E3s for proteasomal degradation. *Journal of Biological Chemistry* **278**, 43169–43177.
119. Naramura, M., Kole, H.K., Hu, R.J. *et al.* (1998) Altered thymic positive selection and intracellular signals in Cbl-deficient mice. *Proceedings of the National Academy of Sciences, USA* **95**, 15547–15552.
120. Shahinian, A., Pfeffer, K., Lee, K.P. *et al.* (1993) Differential T cell costimulatory requirements in CD28-deficient mice. *Science* **261**, 609–612.
121. Hanlon, A., Jang, S., Salgame, P. (2005) Cbl-b differentially regulates activation-induced apoptosis in T helper 1 and T helper 2 cells. *Immunology* **116**, 507–512.
122. Gronski, M.A., Boulter, J.M., Moskophidis, D. *et al.* (2004) TCR affinity and negative regulation limit autoimmunity. *Nature Medicine* **10**, 1234–1239.
123. Loeser, S., Loser, K., Bijker, M.S. *et al.* (2007) Spontaneous tumor rejection by cbl-b-deficient CD8 + T cells. *Journal of Experimental Medicine* **204**, 879–891.
124. Wohlfert, E.A., Gorelik, L., Mittler, R. *et al.* (2006) Cutting edge: deficiency in the E3 ubiquitin ligase Cbl-b results in a multifunctional defect in T cell TGF-beta sensitivity *in vitro* and *in vivo*. *Journal of Immunology* **176**, 1316–1320.
125. Yokoi, N., Komeda, K., Wang, H.Y. *et al.* (2002) Cblb is a major susceptibility gene for rat type 1 diabetes mellitus. *Nature Genetics* **31**, 391–394.
126. Yokoi, N., Namae, M., Fuse, M. *et al.* (2003) Establishment and characterization of the Komeda diabetes-prone rat as a segregating inbred strain. *Experimental Animals* **52**, 295–301.
127. Bergholdt, R., Taxvig, C., Eising, S. *et al.* (2005) CBLB variants in type 1 diabetes and their genetic interaction with CTLA4. *Journal of Leukocyte Biology* **77**, 579–585.

128. Leng, Q., Borkow, G. and Bentwich, Z. (2002) Attenuated signaling associated with immune activation in HIV-1-infected individuals. *Biochemical and Biophysical Research, Communications* **298**, 464–467.
129. Leng, Q., Bentwich, Z., Magen, E. *et al.* (2002) CTLA-4 upregulation during HIV infection: association with anergy and possible target for therapeutic intervention. *AIDS* **16**, 519–529.
130. Babu, S., Blauvelt, C.P., Kumaraswami, V. *et al.* (2006) Regulatory networks induced by live parasites impair both Th1 and Th2 pathways in patent lymphatic filariasis: implications for parasite persistence. *Journal of Immunology* **176**, 3248–3256.
131. Balachandran, P., Dragone, L., Garrity-Ryan, L. *et al.* (2007) The ubiquitin ligase Cbl-b limits Pseudomonas aeruginosa exotoxin T-mediated virulence. *Journal of Clinical Investigation* **117**, 419–427.
132. Chiang, J.Y., Jang, I.K., Hodes, R. *et al.* (2007) Ablation of Cbl-b provides protection against transplanted and spontaneous tumors. *Journal of Clinical Investigation* **117**, 1029–1036.
133. Kosoy, R., Yokoi, N., Seino, S. *et al.* (2004) Polymorphic variation in the CBLB gene in human type 1 diabetes. *Genes and Immunity* **5**, 232–235.
134. Chen, M.L., Pittet, M.J., Gorelik, L. *et al.* (2005) Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals *in vivo*. *Proceedings of the National Academy of Sciences, USA* **102**, 419–424.

Abstract

The E3 ubiquitin ligase Cbl-b, a member of the the family of the Cbl (Casitas B-lineage lymphoma) proteins, has been established as an important negative regulator of lymphocyte signalling. Genetic inactivation of Cbl-b revealed it to be a key autoimmunity gene. Cbl-b functions as a gatekeeper in T cell activation that controls activation thresholds and the requirement for co-stimulation. Importantly, Cbl-b deletion uncouples T cell activation from the requirement of CD28 co-stimulation, impairs anergy induction and reduces the sensitivity to regulatory T cell suppression. In consequence, Cbl-b-deficient animals display enhanced T cell proliferation and enhanced susceptibility to autoimmune diseases in various models *in vitro* and *in vivo*.

Key words: Cbl-b; T cell; tolerance; autoimmunity; TCR signalling; co-stimulation

6

Indoleamine 2,3-dioxygenase: transcriptional regulation and autoimmunity

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6.1 Introduction

Indoleamine 2,3-dioxygenase (IDO) is a haem-containing enzyme that catalyses the oxidative cleavage of the indole ring in L-tryptophan (L-Trp), regulating this amino acid catabolism at the initial, rate-limiting level in a specific pathway. L-Trp, the least abundant of the essential amino acids, is utilized for protein synthesis and as a precursor of the neurotransmitter serotonin, yet the majority of the dietary intake is degraded along the so-called kynurenine pathway (named after the first metabolite, L-kynurenine), eventually leading to the biosynthesis of NAD^+. However, the same metabolic pathway, with initial formation of L-kynurenine, can be activated by two distinct, poorly homologous ($\approx$12%) enzymes: IDO and tryptophan 2,3-dioxygenase (TDO).

TDO is a constitutive enzyme restricted to the liver, whereas IDO is an inducible, intracellular enzyme widely expressed in several organs, and in particular in the lung, small and large intestine, spleen, kidney, stomach, brain and placenta. Remarkable expression of IDO is found in myeloid lineage cells (dendritic cells (DCs), monocytes, macrophages, eosinophils), epithelial cells, fibroblasts, vascular smooth muscle and endothelial cells, and in certain tumours as well. The two enzymes differ in substrate specificity. TDO is highly specific for the L-isomer of Trp, whereas IDO is capable of oxidizing a broad range of substrates, including not only L-Trp, but also D-Trp,

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tryptamine and serotonin [1,2]. Very recently, a third murine and human enzyme with similar tryptophan-degrading potential has been found, indoleamine 2,3-dioxygenase-like protein 1 (INDOL1). The highest expression of INDOL1 is in the kidney (with prominent localization in tubular cells), followed by the epididymis, liver and spermatozoa. Preliminary evidence suggests that INDOL1 has the same activity as IDO, but it is still unclear whether the former possesses additional functions [3].

IDO is encoded by a gene located on chromosome 8, both in humans (*INDO*) and mice (*Indo*) [4]; in the two species, 57% identity in the primary sequence is observed for this gene [5]. Induced at the transcriptional level by inflammatory stimuli (most notably interferon-γ, IFN-γ), the mature form of IDO is a monomeric haem-containing protein of approximately 45 kDa, folded in two distinct α-helical domains, with the haem prosthetic group positioned between those two domains [6]. IDO can be expressed in a cell with no functional activity, and several putative mechanisms control its activation, including post-translational modifications, alternative splicing and the presence of co-factors (i.e. a haem group and reactive oxygen species).

IDO functional activity is lost in the presence of competitive inhibitors, such as 1-methyl-tryptophan, which binds the active site of the enzyme with high affinity and results in long-lasting impairment of enzyme activity. The catalytic function of IDO requires a functional haem group. When purified in an inactive state in its ferric form (Fe^{3+}), IDO activation requires the reduction of the ferric to ferrous iron, which enables binding of L-Trp and O_2 to the enzyme active site and the subsequent oxidation of the amino acid pyrrole ring to *N*-formyl-kynurenine (which, in turn, deformylates to L-kynurenine). The intracellular co-factors required for IDO haem-iron reduction are incompletely understood. Putative candidates include superoxide anion, dihydroflavin mononucleotide, tetrahydrobiopterin and cytochrome reductases [2,7]. Because IDO consumes superoxide for the reduction of the inactive ferric to the active ferrous form, the enzyme might work as an antioxidant [8]. In this regard, clearance of radical species by IDO and impaired conversion of tryptophan to serotonin (with vasoactive properties) could represent protective mechanisms in inflammatory states [9]. However, because in the intracellular environment the haem ferrous form is easily oxidized to the ferric form again, thus releasing superoxide anion, the antioxidant activity of IDO is still a matter of debate [2]. However, in the course of an inflammatory response – when superoxide anion is liberated – superoxide does serve as an activating agent, in that it controls the shift between the inactive and active forms of the enzyme [10].

6.2 L-Trp degradation along the kynurenine pathway and immune functions of IDO

IDO-mediated degradation of tryptophan initiates an enzymic pathway, resulting in the production of various immunoregulatory and neuroactive metabolites [4,7,9]. The first step in this pathway (Figure 6.1) consists of the conversion of L-Trp to

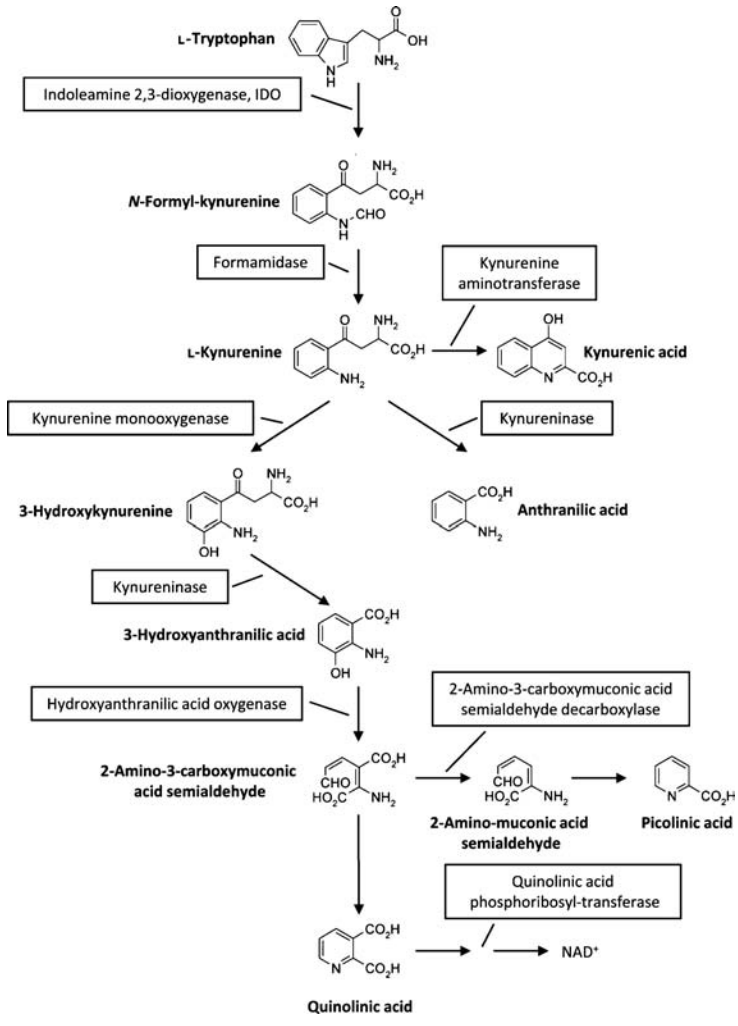

Figure 6.1 The enzymic pathway of tryptophan catabolism. IDO catalyses tryptophan transformation into L-kynurenine at the initial, rate-limiting step of an enzymic cascade, named the kynurenine pathway after its first metabolite. Several downstream enzymes participate in the subsequent production of tryptophan catabolites, which possess biological activity and are collectively called kynurenines.

N-formyl-kynurenine, further degraded to L-kynurenine by formamidase. L-Kynurenine subsequently serves as a substrate for a distinct set of enzymes. Kynureninase gives rise to anthranilic acid, kynurenine monooxygenase yields 3-hydroxy-kynurenine and kynurenine aminotransferases produce kynurenic acid by the irreversible transamination of L-kynurenine. 3-Hydroxykynurenine is converted to 3-hydroxyanthranilic acid by kynureninase. 3-Hydroxyanthranilic acid oxygenase catalyses the

conversion of 3-hydroxyanthranilic acid to 2-amino-3-carboxymuconic acid semialdehyde, which either rearranges non-enzymically to form quinolinic acid or serves as a substrate for 2-amino-3-carboxymuconic acid semialdehyde decarboxylase, leading to the production of picolinic acid. Quinolinic acid phosphoribosyltransferase catalyses the conversion of quinolinic acid to nicotinic acid mononucleotide, further degraded to NAD^+. The activation of this pathway, controlled by IDO at the initial level, leads to L-Trp depletion in local tissue microenvironments and promotes formation of bioactive metabolites (collectively called kynurenines). Both events, which depend on IDO, play a crucial role in host innate immune defence and in the control of adaptive immunity.

Host defence

The finding that IDO is expressed in the lung during either bacterial endotoxic shock or influenza viral infection [11] suggested that IDO plays a role in the defence against various types of microbial infection. IDO has been shown to inhibit the *in vitro* replication of a wide range of intra- and extracellular organisms, from protozoa to viruses (i.e. cytomegalovirus and herpes simplex virus), via L-Trp deprivation [12]. Although most microorganisms can synthesize their own tryptophan, some depend on an exogenous source of the amino acid (auxotrophs), and are sensitive to the activity of IDO. *Chlamydia pneumoniae*, *Toxoplasma gondii*, group B Streptococci and Mycobacteria are examples of auxotrophic microorganisms amenable to IDO-mediated tryptophan starvation, in which pathogen or viral replication is restored by externally added tryptophan.

This confirms that specific IDO effects are related to its ability to deplete this essential amino acid [13–17]. Additional evidence supporting the anti-microbial role of IDO comes from an increased expression of the enzyme *in vivo* in response to infectious agents. Nevertheless, the putative anti-microbial role of IDO has become increasingly difficult to reconcile with – or separate from – its emerging role as a major negative regulator of innate and adaptive immune responses.

Immune tolerance

A major feature of the immune system is the ability to discriminate between self and non-self and to activate either tolerogenic or immunogenic programs, whose molecular mechanisms are complex and incompletely understood. Among cells of the immune system, DCs are key regulators of immune outcomes, capable of promoting or suppressing T cell responses depending on environmental factors [18]. As proven by numerous pieces of evidence from different settings, one major suppressive mechanism mediated by IDO is the expression of a tolerogenic potential in particular subsets of DCs, either possessing the functionally active enzyme or amenable to IDO induction by appropriate stimuli (as is the case for plasmacytoid DCs).

A critical role for IDO in the maintenance of tolerance was originally demonstrated by Munn *et al.* in 1998 [19]. Studying allogeneic fetus rejection in pregnant mice, they proposed that IDO-dependent tryptophan depletion limits local availability of the amino acid to proliferating T cells and thus prevents rejection of the fetus. This study outlined the importance of IDO in fetal/maternal tolerance and suggested L-Trp deprivation as the principal IDO-dependent mechanism of T cell inhibition [20–22]. However, growing evidence points to a multiplicity of IDO effects and to a synergistic activity of local tryptophan depletion and the production of several immunomodulatory kynurenines. This accounts for an overall complex immunosuppressive effect, with different major components in different settings, each component targeting a specific T cell or non-T cell subset [4,23].

IDO and the downstream enzymes in the kynurenine pathway produce a pool of tolerogenic tryptophan metabolites. Recent studies have shown that cell proliferation, activation and/or survival can be directly suppressed by several kynurenines, such as L-kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and quinolinic and picolinic acids [24–31]. In particular, these molecules appear to specifically inhibit the proliferation of activated T cells, of both $CD4^+$ and $CD8^+$ subsets, rendering them unresponsive to further stimulation. The same molecules are yet ineffective on resting T cells [25–27,29].

Induction of cell death [25,28,30] and cell-cycle arrest [26,27] have been proposed to contribute to overall T cell suppression. T helper (Th) 1-polarized cells appear to be more susceptible than Th2 cells to kynurenine-mediated apoptosis *in vitro*, although Th2 cells are specifically targeted by IDO for inhibition of self-expansion and activation [32,33]. The tolerogenic role of tryptophan metabolites as a whole is further supported by evidence that even in the absence of functional IDO and with no tryptophan depletion, the downstream enzymes in the kynurenine pathway mediate induction of T cell death and tolerogenesis in the presence of externally added kynurenine-type substrates [30].

Activation of the cell response to stress

Another mechanism contributing to the tolerogenic effect of tryptophan catabolism is activation of the cell response to amino acid deficiency. In the microenvironment of DCs expressing functional IDO, and as a result of its activity, a reduction in tryptophan concentration occurs, which causes accumulation of uncharged tRNA in neighbouring T cells. Such increased levels of tRNA represent the signal for activation of the amino acid-sensitive so-called general control non-derepressible 2 (GCN2) stress-kinase pathway [34,35]. GCN2 initiates a stress-response programme that triggers cell-cycle arrest and anergy induction in responding T cells, but also differentiation, adaptation or apoptosis depending on cell type [36]. GCN2 kinase is required by $CD8^+$ T cells to sense, and respond to, tryptophan depletion mediated by IDO. T cells lacking GCN2 proliferate normally in the presence of IDO^+ DCs and are not susceptible to IDO-induced anergy [34]. The GCN2 kinase pathway has also been shown to be involved

in the early downregulation of the T cell receptor (TCR) ζ chain in $CD8^+$ T cells, which results in an impaired cytotoxic activity [35]. Remarkably, the cooperative effects of tryptophan starvation and immunoactive kynurenines can induce autoimmune-preventive $CD4^+$ regulatory T cells (Tregs), capable of effectively controlling pathogenic T cells *in vivo* [35].

Induction of Tregs

The expression of functionally active IDO differs in several subsets of DCs and depends on environmental stimuli. IFN-γ, a pro-inflammatory cytokine, could contribute to T cell homeostasis by promoting activation-induced cell death and the generation of Treg cells, both of these events being crucial to the maintenance of peripheral tolerance. Recent evidence suggests a self-amplifying suppressive loop between IDO^+ DCs, which induce Treg function, and Treg cells, which act directly on tolerogenic DCs to promote autocrine activation of the IDO mechanism [37]. As a matter of fact, Treg cells constitutively express cytotoxic T lymphocyte antigen 4 (CTLA-4), which has a key role in T cell-mediated immunological tolerance [38]. It is commonly believed that CTLA-4 functions through a combination of inhibitory T cell signalling and blockade of the CD28/B7 co-stimulatory pathway, which is instrumental in T cell activation, expansion, differentiation and prevention of anergy. It has been proposed that CTLA-4 expressed by Treg cells – upon binding its B7-1 receptor on the surface of DCs – triggers a so-called reverse signalling event in DCs, which causes the release of IFN-γ, in turn capable of inducing IDO at the transcriptional level [37,39]. Thus, this self-amplifying regulatory network involves interactions between IDO-competent DCs and Tregs.

Neuroactive kynurenines

In addition to their immunomodulatory function, selected tryptophan metabolites have also neuroactive properties. Among these, the best characterized is quinolinic acid, because of its neurotoxic effect when used *in vivo* by direct intracerebral injection [40] or *in vitro* on cultured neural cells [41]. Quinolinic acid activates the subpopulation of neural glutamate receptors sensitive to *N*-methyl-D-aspartate (NMDA), inducing apoptosis and neuronal necrosis [42]. The tight correlation of quinolinic acid levels in cerebrospinal fluid with the severity of the neurological deficits has suggested that the molecule plays a direct role in the pathogenesis of neurodegenerative disorders, such as poliovirus brain infection [43], cerebral malaria [44], ischaemic brain disease [45] and the acquired immunodeficiency dementia complex caused by an immunodeficiency virus type I infection [46]. The production of quinolinic acid closely reflects the local induction of IDO during inflammatory conditions in the central nervous system, and its accumulation, subsequently, results in neurodegeneration. It has been suggested that, in the course of neuroinflammatory processes observed in many neurodegenerative disorders (including Parkinson's and Alzheimer's diseases)

IFN-γ-mediated induction of IDO in microglia can initiate two counteracting effects: a tolerogenic, protective negative-feedback loop which downmodulates neuroinflammation, and a neurotoxic action depending on the overproduction of quinolinic acid, with the latter effect prevailing on the former for the fatal evolution of the pathology [47].

Alzheimer's disease is the most common form of dementia around the world. Although its precise aetiology is still unknown, it has been recently demonstrated that the kynurenine pathway is upregulated in Alzheimer's disease brains, thus leading to increased neurotoxic quinolinic acid [48]. An immune activation, including increased production of pro-inflammatory cytokines and activation of IDO, has been described in major depression as well. In this pathology, an enhanced consumption of serotonin and its precursor, tryptophan – owing to sustained IDO activation – could well explain the reduced availability of serotoninergic neurotransmission [49]. Nevertheless, neuroactive kynurenines, besides their role in the pathogenesis of some central nervous system diseases, likely have a physiological function as well, with tryptophan metabolites acting as agonist or antagonist ligands at neurotransmitter receptor sites and contributing to neural growth and synaptogenesis.

6.3 IDO immunobiology and therapeutic intervention

IDO plays an important physiological role in the defence mechanisms against a variety of infectious pathogens, as well as in regulating the immune response against self and non-self antigens. These findings suggest that a dysfunctional IDO could represent a key component in the generation of pathological conditions, both strictly immune and non-immune in nature. Yet, IDO may represent a therapeutic target as well. For example, aberrant IDO expression has the potential to block the host's protective response to tumour antigens [50,51], to inhibit the ability of activated T cells to kill tumour cells [52] and to enhance the suppressive activity of Treg cells, both at the tumour site and in lymph nodes. On the other hand, loss of tolerance to self, due to defective IDO activity, leads to exaggerated activation of the inflammatory and immune systems, causing tissue damage and often resulting in allergic, inflammatory or autoimmune disorders [32,53–55]. Therefore, IDO could be considered an ideal target for therapeutic strategies: inhibition of IDO in tumours may attenuate the ability of tumours to evade the immune surveillance [20]; in contrast, drugs enhancing or mimicking IDO activity may be beneficial during autoimmune or inflammatory diseases [27,55].

6.4 Transcriptional regulation of the IDO-encoding gene

Whereas the enzymic activity of IDO has largely been characterized, the transcriptional regulation of *INDO* is currently under intense investigation. *Indo*, encoding

the IDO protein, is an inducible gene almost ubiquitously expressed at low levels. Nevertheless, a two-step regulation, first transcriptional and then post-translational, seems to be mandatory for the acquisition of full IDO competence by the cell [56]. Murine and human IDO genes are both located on chromosome 8 in a tandem arrangement together with the recently described *INDOL1* gene. Transcription of *INDO* is restricted to a series of upstream regulatory elements located on its promoter. Historically, induction of IDO was first ascribed to the activity of IFN-γ [57], which is still considered the most important inducer of *INDO*. The IFN-γ-dependent expression of this gene depends on the presence of an IFN-γ-activated site (GAS) and two IFN-stimulated response elements (ISREs). Signal transducer and activator of transcription 1 (STAT1) acts directly through binding of GAS and indirectly through induction of IFN-regulatory factor (IRF)-1, which binds ISREs. Earlier studies [58] established that the GAS and ISRE sequences could cooperate for the expression of *Indo* in response to IFN-γ. Subsequently, both GAS and ISRE have been found to be crucial for expression of *Indo* as mediated by STAT3 [59] or IFN-regulatory factor-8 (IRF-8) [60]. Nuclear factor κB (NF-κB) also contributes to this effect by binding a GAS/κB site, which combines a GAS element with a non-consensus site for NF-κB. Non-canonical NF-κB was recently indicated as being responsible for the induction of IDO expression in plasmacytoid DCs (pDCs) in response to reverse signalling by a soluble form of glucocorticoid-induced TNF receptor-related protein (GITR-Ig) [32]. Both the murine and human promoters of *INDO* contain a putative partial binding site, recognized by the non-canonical NF-κB dimer, p52-REL-B. In contrast, *Bin1*, a BAR-adapter-encoding gene, plays a major role in the negative regulation of *Indo*, affecting its STAT1- and NF-κB-dependent expression [61]. A further repressor of *Indo* transcription is represented by the transmembrane immunoreceptor tyrosine-based activation motif (ITAM) adapter DAP12. DCs from mice bearing non-functional DAP12 constitutively express high levels of an active form of IDO [62]. Moreover, the protein tyrosine kinase-binding protein (*Tyrobp*) gene, coding for DAP12, is negatively modulated by IRF-8 [60], which contributes to IDO modulation by positively regulating its expression and negatively regulating its 'repressor' DAP12. Signals responsible for the induction of IDO expression employ the above-mentioned regulatory elements contained in the *INDO* gene promoter. However, the transcriptional regulation of IDO is complex; it is cell-type specific and it involves the cooperation and integration of different transcription factors, which are activated in the cell in response to extracellular stimuli.

It is possible to distinguish the huge number of regulatory molecules of *INDO* transcription in soluble or membrane-anchored modulators. The former include every soluble molecule with regulatory effects on *INDO* transcription, such as cytokines, hormones, prostaglandins, and Toll-like receptor (TLR) ligands. The latter (B7 co-receptors, CD200R, GITR ligand (GITRL) and CD40) involve cell-contact-dependent stimuli able to induce IDO expression in one of the two interacting cells (Figure 6.2).

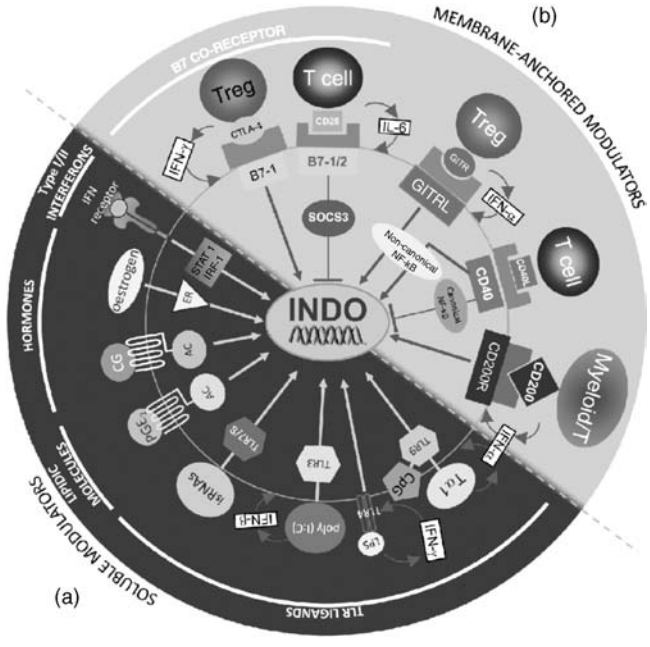

Figure 6.2 A multiplicity of mechanisms involve soluble or membrane-anchored modulators of *INDO* transcription. (a) Soluble modulators include type I/II IFNs acting through their specific receptor via STAT1 and/or IRF-1 activation. Hormones, such as oestrogens and chorionic gonadotropin (CG), and lipidic molecules like prostaglandin E_2 (PGE_2), positively modulate *INDO* transcription via their specific signalling which involves intracellular oestrogen receptors (ER) or activation of adenylate cyclase (AC), respectively. TLR ligands through engagement of the specific TLRs can directly, or via autocrine production of type I/II IFNs, modulate *INDO* transcription. (b) Membrane-anchored modulators include the B7 coreceptors CTLA-4 and CD28, expressed on Treg cells and naïve T cells, respectively. CTLA-4 positively modulates *INDO* transcription via autocrine IFN-γ and CD28 can repress *INDO* transcription through autocrine IL-6 production and SOCS3 upregulation in the cell. GITR, expressed on Treg cells, positively modulates *INDO* transcription by activating non-canonical NF-κB through its specific co-receptor GITRL. Engagement of CD40 by CD40L-expressing T cells results in either canonical or non-canonical NF-κB activation, which can diametrically modulate *INDO* transcription. CD200-expressing cells induce *INDO* transcription via CD200R engagement and release of IFN-α.

Modulators of INDO transcription

Type I/II IFNs represent the most important cytokines that induce *INDO* transcription. Although IFN-γ (type II IFN) is considered the main mediator of IDO induction, IFN-α and IFN-β (type I IFNs) can positively regulate its transcription as well by acting on the two ISREs located on the *Indo* promoter. Depending on the cell type, IFN-α is up to three orders of magnitude less potent at inducing IDO expression than IFN-γ [12]. However, in pDCs, type I and type II IFNs are roughly equipotent and exert additive,

perhaps synergistic, effects. A current paradigm of IDO induction in pDCs may be that it can be activated by ligands (e.g. CpG-rich oligodeoxynucleotides (CpG-ODNs), CTLA-4-Ig, CD200-Ig and GITR-Ig) that induce pDC-derived production of either cytokine. Interestingly, in a minor population of mouse splenic $CD19^+$ DCs, IDO was recently identified as an essential triggering mechanism that positively regulates the downstream production of IFN-α [63]. Through a positive-feedback loop, this mechanism enables an immediate amplification of the initial *Indo* transcription. Therefore, IDO could locally enhance its own production through IFN-α, which represents both the initial inducer of *Indo* transcription and the downstream mechanism of IDO. This mutual regulation of IDO and IFN-α in $CD19^+$ DCs contributes to a balanced stimulation of both protective inflammatory and anti-inflammatory effects of tryptophan catabolism.

Although *Indo* is strictly an IFN-γ-inducible gene, tumour necrosis factor α (TNF-α) can synergistically increase its transcription together with IFN-γ. Nuclear concentrations of STAT1 and IRF-1, which respectively bind GAS and ISRE regulatory sequences, were found to increase after stimulation with IFN-γ and TNF-α, relative to stimulation with individual cytokines. Moreover, by limiting NF-κB nuclear translocation, the synergistic increase of *Indo* transcription was blocked, suggesting that NF-κB activation is required for the cooperative IDO induction in response to IFN-γ and TNF-α [64]. Fujigaki *et al.* described a STAT1-/IRF-1-independent induction of IDO gene, which involves p38 mitogen-activated protein kinase (MAPK) and the NF-κB pathway in THP-1 cells stimulated with pro-inflammatory cytokines, such as TNF-α, interleukin-1β (IL-1β) and IL-6 [65]. Earlier studies described the abrogation of IFN-γ-stimulated expression of IDO by transforming growth factor β (TGF-β) in human skin and synovial fibroblasts. TGF-β did not interfere with STAT1- or IRF-1-binding activity to the *INDO* promoter, but the stability of IDO mRNA was reduced in fibroblasts treated with TGF-β [66]. Recently, impaired IFN-γ-induced IDO expression was found to characterize primary biliary cirrhosis, an autoimmune chronic cholestatic liver disease. Primary biliary cirrhosis was correlated with an increased frequency of a gain-of-function single nucleotide polymorphism within the TGF-β promoter region, a molecule known to suppress *INDO* transcription [67].

An emerging class of soluble inducers of IDO expression is represented by hormones, produced at high levels during pregnancy, which induce a state of immunological tolerance related to temporal remission of pre-existing autoimmune disorders. A key pregnancy hormone is human chorionic gonadotropin, whose administration to non-obese diabetic (NOD) mice inhibits the activation of diabetogenic T cells and the progression of type 1 diabetes, by upregulating the expression of the IDO gene in DCs [68]. Furthermore, oestrogen-dependent induction of IDO was reported in human DCs from patients with multiple sclerosis, supporting the hypothesis that the reduction in number of multiple sclerosis relapses observed during pregnancy may be related to an oestrogen/IDO axis [69].

Prostaglandins (PGs), a family of arachidonate-derived molecules released during inflammation, display immunomodulatory properties in several models. Maturation of DCs in the presence of PGE_2 results in IDO mRNA increase; however, a second signal

via TNF-α or TLRs is required to express an active form of the enzyme. The effect of PGE_2 is mediated by activation of adenylate cyclase via the G_s protein-coupled receptor E prostanoid-2 [56]. Importantly, different carcinomas associated with high levels of PGE_2 express increased amounts of IDO in peritoumoral DCs. Therefore PGE_2, by upregulating IDO expression, may be suggested to be a mediator of early events during the onset of immune tolerance in cancer [70].

IDO gene expression increases in inflammatory conditions with the aim of limiting this response. Therefore, a further class of pro-inflammatory modulators of *Indo* expression is represented by TLR ligands. A series of recent studies has demonstrated that the administration of CpG-ODNs, all TLR9 ligands, may induce *Indo* transcription in several experimental settings [71]. TLR9 is expressed in different cell types and CpG-ODN ligation leads to the induction of pro-inflammatory cytokines (type I IFN) and membrane-anchored ligands (CD40, CD80, CD86), which may be, in turn, responsible for IDO expression. Systemic administration of relatively high doses of CpG-ODN to mice was found to induce IDO expression in a minority of splenic DCs through IFN-α-dependent activation of STAT1 [72]. Studies with knockout mice demonstrated that CpG-ODN-induced IDO expression was dependent on TLR9, but independent of type I and II IFNs [73]. Moreover, through IRF-8, TLR9 signalling can activate NF-κB in murine DCs and, interestingly, the *Indo* promoter also contains a critical NF-κB-binding site [74]. Whatever mechanisms are involved in CpG-ODN-induced IDO expression, these studies offer novel opportunities for the use of CpG-ODNs as immunosuppressive agents.

A further TLR9 ligand is represented by thymosin α1, a naturally occurring thymic peptide. Recently, thymosin α1 was found to induce IDO expression in human and murine DCs via TLR9 and type I IFN receptor signalling [75]. Like TLR9 ligands, the TLR4 ligand lipopolysaccharide (LPS) can induce IDO expression. A series of studies aimed at clarifying whether LPS-induced IDO expression is mediated by IFN-γ. Recent observations indicate that LPS can induce IDO expression via an IFN-γ-independent mechanism, involving phosphoinositide-3-kinase and c-Jun N-terminal kinase activation [76]. Besides the more extensively studied TLR9 and TLR4 ligands, other TLR ligands have been indicated as inducers of *INDO* transcription. The TLR3 ligand poly (I:C) results in IDO expression in human astrocytes, in part via IFN-β, but not IFN-γ, and both NF-κB and IRF-3 are required [77]. Moreover, specific ligands of TLR7/8 can induce IDO expression in human monocytes, but not in monocyte-derived DCs. IDO induction by TLR7/8 ligands seems to be lost when monocytes are programmed to differentiate into DCs [78]. Although induction of IDO expression by different TLR ligands has been studied extensively, which TLR signals are mainly required for IDO induction is still unclear.

Membrane-anchored modulators of INDO transcription

Many membrane-anchored modulators of *INDO* transcription mainly act by inducing the production of cytokines such as type I/II IFNs, as illustrated above. However, it is

of interest to further expand on this class of *INDO* transcription regulators by highlighting their physiological properties. Interestingly, the inhibitory receptor CTLA-4, constitutively expressed on activated T cells or Tregs, was reported to be sufficient to induce IDO expression in human and murine DCs through reverse signalling involving B7-1 (CD80) molecules [39]. Although this mechanism is IFN-γ-dependent, soluble CTLA-4 was also observed to induce *Indo* transcription in DCs from NOD female mice, which are characterized by an impaired IDO expression in response to IFN-γ. Soluble CTLA-4 can indeed bypass IFN-γ unresponsiveness – hence defective expression of *Indo* – through the concomitant activation of FOXO3a, a member of the forkhead box O (FOXO) family of transcription factors, and the induction of antioxidant effects [54]. Bidirectional signalling along the B7/CTLA-4 co-receptor axis enables reciprocal conditioning of T cells and DCs. Moreover, ligation of B7 (CD80 and CD86) molecules by the stimulatory receptor CD28, constitutively expressed on naïve T cells, was shown to exert an effect on *Indo* induction comparable to that of CTLA-4 ligation, when suppressor of cytokine signalling 3 (SOCS3) was silenced by small interfering RNA in murine DCs [79]. It has been suggested that in absence of SOCS3 IFN-γ signalling may be released from a critical inhibitory control exerted by SOCS3, leading to induction of IDO expression. In addition, IL-6, produced by DCs upon B7–CD28-Ig interaction [80], might contribute to IDO expression by virtue of its ability to trigger IFN-γ-like responses [81].

While IFN-γ appears to be the major cytokine responsible for B7-mediated *INDO* transcription, type I IFN mediates IDO gene induction in murine pDCs in response to engagement of CD200R1 receptor by CD200-Ig, the soluble form of CD200 [82], a broadly distributed cell-surface glycoprotein highly expressed on myeloid and T cells. Moreover, CD200-Ig provided protection in an autoimmunity model of collagen-induced arthritis in mice [83], indicating that CD200 is a tolerance signalling molecule the downstream cellular events of which could be represented by IDO expression. Emerging data indicate IDO expression as being the most important mechanism characterizing bidirectional conditioning of antigen-presenting cells and T cells. Recently, further evidence of such IDO-mediated conditioning was observed in pDCs in response to GITR-Ig [32]. The Treg-expressed receptor GITR activates *Indo* transcription in pDCs through a mechanism of reverse signalling via its specific ligand GITRL. GITRL-mediated induction of IDO requires the activation of the non-canonical pathway of NF-κB and the production of IFN-α. In contrast to the pro-inflammatory signals triggered by the canonical NF-κB pathway, emerging data indicate this 'alternative' pathway as being responsible for opposite roles, leading to resolution of inflammatory processes [84] and establishment of self-tolerance [85]. Therefore, the GITR/GITRL co-receptor system, by activating non-canonical NF-κB pathway, which leads to IDO induction, may represent a homeostatic mechanism limiting exacerbation of the inflammatory process in pDCs, potent activators of innate immunity. Notably, CD40 signalling, which activates both canonical and non-canonical NF-κB [86], has been found to diametrically modulate IDO, depending on environmental factors [87,88]. Indeed, ligation of CD40 on DCs induces an early expression of inflammatory mediators requiring canonical NF-κB pathway, followed

by later expression of anti-inflammatory IDO, which requires non-canonical NF-κB signalling [89]. Moreover, treatment with CD40-Ig results in indefinite allograft survival in a model of heart allograft in the rat [90]. Allospecific $CD8^+$ Treg cells purified from CD40-Ig-treated animals were responsible for maintaining allograft survival by inducing IDO expression in graft endothelial cells via IFN-γ. These data suggest that IDO expression by components of non-canonical NF-κB signalling participates in a balance with canonical NF-κB and in the control of inflammatory processes. This may contribute to contrasting the onset of autoimmunity.

6.5 Impaired IDO activity and loss of tolerance in autoimmune diseases

Immune-system homeostasis is guaranteed by a balance between tolerance against self and activation of the immune responses. When such a balance fails with a prevalence of the latter, autoimmune disorders arise, leading to cell and tissue destruction which results in chronic and invaliding diseases. Autoimmune disorder origins reside both in excessive T cell activation by autoantigens and in defective negative modulation of immune responses, which involves the emerging immunoregulatory role of IDO. These two distinct pathways involve the actions of different cell types, genes and soluble mediators, all concurring to a common final effect; that is, deregulation of so-called physiological immunity and the onset of an autoimmune state. Until a few years ago the Th1 subset and associated cytokines were thought to be principally responsible for the development of autoimmune responses [91]. The recent discover of Th17 cells has allowed us to reconsider the specific roles of $CD4^+$ T cell pro-inflammatory subsets. Naïve Th0 cell differentiation towards a certain phenotype is critically conditioned by a specific cytokine milieu: IFN-γ-mediated STAT1 signalling leads to Th1 cells through T-bet induction; both TGF-β and IL-6 synergistically induce the expression of the orphan nuclear receptor RORγt (a transcription factor for Th17 lineage specification) and drive the generation of IL-17-producing T cells, whose expansion and survival is sustained by IL-23; on the other hand, TGF-β alone is responsible for the induction of forkhead box P3 (Foxp3) and the differentiation into Treg cells. The tight correlation between the response to a certain cytokine and the polarization to the corresponding Th phenotype has revealed – by means of IFN-γ- or IFN-γ-receptor-deficient mice – that the loss of IFN-γ signalling does not confer resistance to autoimmunity, but renders such mice even more susceptible to autoimmunity [92]. Therefore, the Th1 subset is unlikely to be the only responsible for pathogenetic responses against self. As a matter of fact, ample evidence obtained with p19-deficient (and therefore IL-23-deficient) mice rather suggests that IL-17-producing T cells are capable of inducing organ-specific autoimmunity [93,94]. The pathogenetic events driven by Th17 cell activation can be initially controlled by the contribution of IDO activity. Tryptophan catabolites have recently been shown both to suppress RORγt transcription factor and to induce *Foxp3* expression, resulting in inhibition of Th17 cells and in Treg generation, respectively [95,96]. Thus, in a fungal

infectious model, where the Th17 cells play an inflammatory role previously attributed to uncontrolled Th1 cell responses, the capacity of Treg cells to inhibit antifungal immunity, including functional Th17 antagonism, is an IDO-mediated effect resulting in protective tolerance to fungi.

In addition, the maintenance of peripheral tolerance operated by IDO is crucial for contrasting and avoiding the onset of autoimmune disorders, as found in settings where self-tolerance has already been disrupted, and pharmacological inhibition of IDO causes disease worsening in experimental autoimmune encephalomyelitis [47], allergic asthma [97] and inflammatory bowel disease [98].

Autoimmune diabetes

In type 1 diabetes the destruction of insulin-producing β cells in pancreatic islets of Langerhans is a T cell-mediated autoimmune process [99]. The pathogenic evolution of this self-reactive disorder is related to impaired IDO function in DCs, which become unresponsive to the tolerogenic effect of IFN-γ, as documented in a study on NOD mice, a widely used animal model for type 1 diabetes [53]. In particular, the overproduction of peroxynitrite in prediabetic female NOD mice resulted in blockade of the STAT1 pathway, and therefore of IFN-γ-dependent IDO induction. However, the impaired tryptophan catabolism of those DCs could be restored by treating the cells *in vitro* with a soluble form of CTLA-4 (CTLA-4-Ig), capable of preventing peroxynitrite formation via activation of FOXO3a transcription factor and subsequent superoxide dismutase induction [54]. Furthermore, CTLA-4-Ig is effective in activating IDO in DCs when administrated *in vivo* in mice with chemically induced diabetes, and it is responsible for a potent immunosuppressive action which enables long-term engraftment of pancreatic islet allografts [39]. Similar tolerogenic, IDO-mediated protective effects are also displayed by Treg membrane-anchored CTLA-4 in an experimental setting of diabetes transfer. Those Tregs, generated *in vitro* by a low concentration of tryptophan and a mixture of kynurenines, protect mice from fulminant diabetes when co-transferred with diabetogenic NOD/BDC2.5 splenocytes into NOD/SCID mice [35]. These and other results suggest that local modulation of tryptophan catabolism may be instrumental in facilitating islet transplantation as a therapy for type 1 diabetes. Thus immunotherapy targeting suppression of established β-cell autoimmunity, involving antigen-based as well as non-antigen-specific (systemic) treatments, or availing the combined expression of selected genes (IDO, Mn-superoxide dismutase and insulin-regulated aminopeptidase) in transplanted islets (to more efficiently protect their grafts from diabetogenic T cells [100]) might beneficially modify immunity through the induction of Treg cells and successfully maintain or re-establish tolerance in type 1 diabetes.

Experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis, is a T cell-mediated demyelinating disease of the central nervous system, differently

regulated by IDO, in the preclinical, acute and remission phases [101]. It has been proposed that autoimmune inflammation during EAE and multiple sclerosis may be self-limited at an early stage by the activation of a negative-feedback loop involving IFN-γ produced by encephalitogenic Th1 cells, local IDO expression and production of T cell-apoptotic tryptophan metabolites [47]. However, this IDO-mediated control can become insufficient, leading to worsening of the disease. The ensuing autoimmune neuroinflammation can be contrasted by an orally active synthetic derivative of anthranilic acid (a tryptophan metabolite) [27], or by the injection of IFN-γ-conditioned (and therefore IDO-competent) DCs [102], while it is exacerbated by administration of 1-methyl-tryptophan [47]. Supported by the further observation of an increased resistance of DAP12 loss-of-function mutant mice to EAE (DAP12 is an adapter molecule negatively regulating IDO activity) [62], these findings highlight the crucial role of IDO in this autoimmune pathology and the therapeutic potential of reinforcing IDO activity in EAE.

Rheumatoid arthritis, lupus erythematosus and primary Sjögren's syndrome

As in EAE, also in rheumatoid arthritis [103], systemic lupus erythematosus [104,105] and primary Sjögren's syndrome [106] a decrease of tryptophan and an increase of kynurenine have been observed in comparison with healthy controls. In these rheumatic autoimmune disorders, characterized by a chronic inflammatory state, the apparently dissonant induction of tryptophan degradation has been associated with the progression or additional symptoms of the disease, and might be explained as an attempt to oppose initial autoreactivity. As a matter of fact, synovial T cells of patients with rheumatoid arthritis have been found to be resistant to IDO-mediated deprivation of tryptophan by virtue of an enhanced storage of the amino acid, mediated by tryptophanyl-tRNA-synthetase activity [107]. Although the function of tryptophan degradation in the pathophysiology of autoimmune diseases still remains unclear, the therapeutic efficacy of treatments involving IDO is evident in experimental models such as collagen type II-induced arthritis [108] and experimental autoimmune uveoretinitis [90,109]. In fact, studies demonstrated that the crosslinking of agonistic antibody to 4-1BB, a T cell-co-stimulatory receptor belonging to the TNF receptor superfamily, results in massive antigen-dependent clonal expansion of new $CD11c^+CD8^+$ T cells. IFN-γ produced by these cells, in turn, acts on DCs inducing IDO, whose activity suppresses antigen-specific $CD4^+$ T cells and inhibits the development of chronic inflammation.

6.6 IDO-based therapies for autoimmune disease

Based on the immunosuppressive properties of tryptophan degradation, the main approaches for treatment of autoimmune diseases may include: (1) agents that induce IDO-mediated degradation of tryptophan; (2) administration of genetically modified, or *in vitro*-conditioned DCs, for enhanced tryptophan metabolism (IDO-competent DCs); and (3) treatment with tryptophan catabolites or their synthetic derivatives.

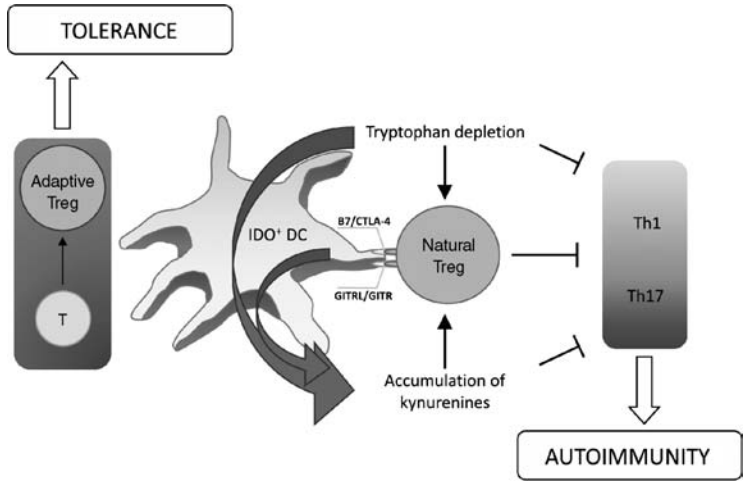

Figure 6.3 IDO-mediated control in the suppression of autoimmune responses and the maintenance of tolerance against self. IDO enzymic activity results in tryptophan deprivation from the cell microenvironment and in local accumulation of catabolites (kynurenines). Both events concur to the *de novo* generation of natural Treg cells, which can directly suppress the development of Th1/Th17 autoimmune response. Moreover, they can trigger a signal in DCs through CTLA-4–B7 and GITR–GITRL interactions capable of increasing IDO activity, further contributing to tryptophan depletion and accumulation of kynurenines, responsible for T cell anergy, cell-cycle arrest and apoptosis. Therefore, DCs expressing a functionally active form of IDO (IDO^+ DCs) play a crucial role in immunological homeostasis, namely the maintenance of a correct balance between tolerance and autoimmunity.

In this regard, the CTLA-4-Ig fusion protein Abatacept™, a potent inducer of IDO expression in DCs, was recently approved by the US Food and Drug Administration for treatment of rheumatoid arthritis [110]. Nevertheless, while transfer of differently modified DCs is still experimental [111], the administration of tryptophan catabolites and their analogues, such as 3,4-dimethoxycinnamoyl anthranilic acid, appears to be a more suitable strategy for treating autoimmune disorders in humans [27].

In conclusion, the crucial role of IDO in tolerance to self, including tryptophan depletion, production of apoptotic catabolites and generation of Tregs (Figure 6.3), represents a promising approach for the development of novel therapeutic strategies to treat autoimmune disorders. An improved understanding of the immunobiology of tryptophan catabolites (e.g. receptors, signalling pathways and cellular responses) will most likely contribute to establishing novel therapeutic options in a wide range of diseases [55].

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References

1. Shimizu, T., Nomiyama, S., Hirata, F. *et al.* (1978) Indoleamine 2,3-dioxygenase. Purification and some properties. *Journal of Biological Chemistry* **253**, 4700–4706.
2. Takikawa, O. (2005) Biochemical and medical aspects of the indoleamine 2,3-dioxygenase-initiated L-tryptophan metabolism. *Biochemical and Biophysical Research, Communication* **338**, 12–19.
3. Ball, H.J., Sanchez-Perez, A., Weiser, S. *et al.* (2007) Characterization of an indoleamine 2,3-dioxygenase-like protein found in humans and mice. *Gene* **396**, 203–213.
4. Mellor, A.L. and Munn, D.H. (2004) IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nature Reviews Immunology* **4**, 762–774.
5. King, N.J. and Thomas, S.R. (2007) Molecules in focus: indoleamine 2,3-dioxygenase. *International Journal of Biochemistry & Cell, Biology* **39**, 2167–2172.
6. Littlejohn, T.K., Takikawa, O., Truscott, R.J. *et al.* (2003) Asp274 and his346 are essential for heme binding and catalytic function of human indoleamine 2,3-dioxygenase. *Journal of Biological Chemistry* **278**, 29525–29531.
7. Thomas, S.R. and Stocker, R. (1999) Redox reactions related to indoleamine 2,3-dioxygenase and tryptophan metabolism along the kynurenine pathway. *Redox Report* **4**, 199–220.
8. Groseclose, E.E. and Frank, L. (1982) The activity of pulmonary indoleamine 2,3-dioxygenase in rats and mice is not altered by oxygen exposure. *Biochimica et Biophysica Acta* **705**, 341–347.
9. Grohmann, U., Fallarino, F. and Puccetti, P. (2003) Tolerance, DCs and tryptophan: much ado about IDO. *Trends in Immunology* **24**, 242–248.
10. Macchiarulo, A., Nuti, R., Bellocchi, D. *et al.* (2007) Molecular docking and spatial coarse graining simulations as tools to investigate substrate recognition, enhancer binding and conformational transitions in indoleamine-2,3-dioxygenase (IDO). *Biochimica et Biophysica Acta* **1774**, 1058–1068.
11. Yoshida, R., Urade, Y., Tokuda, M. *et al.* (1979) Induction of indoleamine 2,3-dioxygenase in mouse lung during virus infection. *Proceedings of the National Academy of Sciences USA* **76**, 4084–4086.
12. Taylor, M.W. and Feng, G.S. (1991) Relationship between interferon-γ, indoleamine 2,3-dioxygenase, and tryptophan catabolism. *FASEB Journal* **5**, 2516–2522.
13. Pfefferkorn, E.R. (1984) Interferon-g blocks the growth of *Toxoplasma gondii* in human fibroblasts by inducing the host cells to degrade tryptophan. *Proceedings of the National Academy of Sciences USA* **81**, 908–912.
14. Carlin, J.M., Borden, E.C., Sondel, P.M. *et al.* (1989) Interferon-induced indoleamine 2,3-dioxygenase activity in human mononuclear phagocytes. *Journal of Leukocyte Biology* **45**, 29–34.
15. Gupta, S.L., Carlin, J.M., Pyati, P. *et al.* (1994) Antiparasitic and antiproliferative effects of indoleamine 2,3-dioxygenase enzyme expression in human fibroblasts. *Infection and Immunity* **62**, 2277–2284.
16. MacKenzie, C.R., Hadding, U. and Daubener, W. (1998) Interferon-γ -induced activation of indoleamine 2,3-dioxygenase in cord blood monocyte-derived macrophages inhibits the growth of group B streptococci. *Journal of Infectious Diseases* **178**, 875–878.
17. Hayashi, T., Rao, S.P., Takabayashi, K. *et al.* (2001) Enhancement of innate immunity against *Mycobacterium avium* infection by immunostimulatory DNA is mediated by indoleamine 2,3-dioxygenase. *Infection and Immunity* **69**, 6156–6164.
18. Moser, M. (2003) Dendritic cells in immunity and tolerance-do they, display opposite functions? *Immunity* **19**, 5–8.
19. Munn, D.H., Zhou, M., Attwood, J.T. *et al.* (1998) Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science* **281**, 1191–1193.
20. Munn, D.H., Sharma, M.D., Hou, D. *et al.* (2004) Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. *Journal of Clinical Investigation* **114**, 280–290.
21. Swanson, K.A., Zheng, Y., Heidler, K.M. *et al.* (2004) CDllc^{+} cells modulate pulmonary immune responses by production of indoleamine 2,3-dioxygenase. *American Journal of Respiratory Cell and Molecular Biology* **30**, 311–318.

22. von Bubnoff, D., Matz, H., Frahnert, C. *et al.* (2002) FcεRI induces the tryptophan degradation pathway involved in regulating T cell responses. *Journal of Immunology* **169**, 1810–1816.
23. Puccetti, P. and Grohmann, U. (2007) IDO and regulatory T cells: a role for reverse signalling and non-canonical NF-κB activation. *Nature Reviews Immunology* **7**, 817–823.
24. Della Chiesa, M., Carlomagno, S., Frumento, G. *et al.* (2006) The tryptophan catabolite L-kynurenine inhibits the surface expression of NKp46- and NKG2D-activating receptors and regulates NK-cell function. *Blood* **108**, 4118–4125.
25. Terness, P., Bauer, T.M., Rose, L. *et al.* (2002) Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. *Journal of Experimental Medicine* **196**, 447–457.
26. Frumento, G., Rotondo, R., Tonetti, M. *et al.* (2002) Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase. *Journal of Experimental Medicine* **196**, 459–468.
27. Platten, M., Ho, P.P., Youssef, S. *et al.* (2005) Treatment of autoimmune neuroinflammation with a synthetic tryptophan metabolite. *Science* **310**, 850–855.
28. Fallarino, F., Grohmann, U., Vacca, C. *et al.* (2002) T cell apoptosis by tryptophan catabolism. *Cell Death and Differentiation* **9**, 1069–1077.
29. Weber, W.P., Feder-Mengus, C., Chiarugi, A. *et al.* (2006) Differential effects of the tryptophan metabolite 3-hydroxyanthranilic acid on the proliferation of human $CD8^+$ T cells induced by TCR triggering or homeostatic cytokines. *European Journal of Immunolology* **36**, 296–304.
30. Belladonna, M.L., Grohmann, U., Guidetti, P. *et al.* (2006) Kynurenine pathway enzymes in dendritic cells initiate tolerogenesis in the absence of functional IDO. *Journal of Immunology* **177**, 130–137.
31. Bosco, M.C., Rapisarda, A., Massazza, S. *et al.* (2000) The tryptophan catabolite picolinic acid selectively induces the chemokines macrophage inflammatory protein-1 a and -1 b in macrophages. *Journal of Immunology* **164**, 3283–3291.
32. Grohmann, U., Volpi, C., Fallarino, F. *et al.* (2007) Reverse signaling through GITR ligand enables dexamethasone to activate IDO in allergy. *Nature Medicine* **13**, 579–586.
33. Hayashi, T., Mo, J.-H., Gong, X. *et al.* (2007) 3-Hydroxyanthranilic acid inhibits PDK1 activation and suppresses experimental asthma by inducing T cell apoptosis. *Proceedings of the National Academy of Sciences USA* **104**, 18619–18624.
34. Munn, D.H., Sharma, M.D., Baban, B. *et al.* (2005) GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. *Immunity* **22**, 633–642.
35. Fallarino, F., Grohmann, U., You, S. *et al.* (2006) The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor z-chain and induce a regulatory phenotype in naive T cells. *Journal of Immunology* **176**, 6752–6761.
36. Harding, H.P., Novoa, I., Zhang, Y. *et al.* (2000) Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Molecular Cell* **6**, 1099–1108.
37. Fallarino, F., Grohmann, U., Hwang, K.W. *et al.* (2003) Modulation of tryptophan catabolism by regulatory T cells. *Nature Immunology* **4**, 1206–1212.
38. Salomon, B. and Bluestone, J.A. (2001) Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. *Annual Review of Immunology* **19**, 225–252.
39. Grohmann, U., Orabona, C., Fallarino, F. *et al.* (2002) CTLA-4-Ig regulates tryptophan catabolism *in vivo*. *Nature Immunology* **3**, 1097–1101.
40. Schwarcz, R., Whetsell, Jr., W.O. and Mangano, R.M. (1983) Quinolinic acid: an endogenous metabolite that produces axon-sparing lesions in rat brain. *Science* **219**, 316–318.
41. Kim, J.P. and Choi, D.W. (1987) Quinolinate neurotoxicity in cortical cell culture. *Neuroscience* **23**, 423–432.
42. Stone, T.W. and Perkins, M.N. (1981) Quinolinic acid: a potent endogenous excitant at amino acid receptors in CNS. *European Journal of Pharmacology* **72**, 411–412.
43. Heyes, M.P., Saito, K., Jacobowitz, D. *et al.* (1992) Poliovirus induces indoleamine-2,3-dioxygenase and quinolinic acid synthesis in macaque brain. *FASEB Journal* **6**, 2977–2989.
44. Sanni, L.A., Thomas, S.R., Tattam, B.N. *et al.* (1998) Dramatic changes in oxidative tryptophan metabolism along the kynurenine pathway in experimental cerebral and noncerebral malaria. *American Journal of Pathology* **152**, 611–619.

45. Saito, K., Nowak, Jr., T.S., Markey, S.P. *et al.* (1993) Mechanism of delayed increases in kynurenine pathway metabolism in damaged brain regions following transient cerebral ischemia. *Journal of Neurochemistry* **60**, 180–192.
46. Heyes, M.P., Brew, B.J., Martin, A. *et al.* (1991) Quinolinic acid in cerebrospinal fluid and serum in HIV-1 infection: relationship to clinical and neurological status. *Annals of Neurology* **29**, 202–209.
47. Kwidzinski, E., Bunse, J., Aktas, O. *et al.* (2005) Indolamine 2,3-dioxygenase is expressed in the CNS and down-regulates autoimmune inflammation. *FASEB Journal* **19**, 1347–1349.
48. Guillemin, G.J., Brew, B.J., Noonan, C.E. *et al.* (2005) Indoleamine 2,3 dioxygenase and quinolinic acid immunoreactivity in Alzheimer's disease hippocampus. *Neuropathology and Applied Neurobiology* **31**, 395–404.
49. Muller, N. and Schwarz, M.J. (2007) Immunological aspects of depressive disorders. *Nervenarzt* **78**, 1261–1273.
50. Staveley-O'Carroll, K., Sotomayor, E., Montgomery, J. *et al.* (1998) Induction of antigen-specific T cell anergy: an early event in the course of tumor progression. *Proceedings of the National Academy of Sciences USA* **95**, 1178–1183.
51. Cuenca, A., Cheng, F., Wang, H. *et al.* (2003) Extra-lymphatic solid tumor growth is not immunologically ignored and results in early induction of antigen-specific T-cell anergy: dominant role of cross-tolerance to tumor antigens. *Cancer Research* **63**, 9007–9015.
52. Gajewski, T.F., Meng, Y., Blank, C. *et al.* (2006) Immune resistance orchestrated by the tumor microenvironment. *Immunological Reviews* **213**, 131–145.
53. Grohmann, U., Fallarino, F., Bianchi, R. *et al.* (2003) A defect in tryptophan catabolism impairs tolerance in nonobese diabetic mice. *Journal of Experimental Medicine* **198**, 153–160.
54. Fallarino, F., Bianchi, R., Orabona, C. *et al.* (2004) CTLA-4-Ig activates forkhead transcription factors and protects dendritic cells from oxidative stress in nonobese diabetic mice. *Journal of Experimental Medicine* **200**, 1051–1062.
55. Romani, L., Fallarino, F., De Luca, A. *et al.* (2008) Defective tryptophan catabolism underlies inflammation in mouse chronic granulomatous disease. *Nature* **451**, 211–215.
56. Braun, D., Longman, R.S. and Albert, M.L. (2005) A two-step induction of indoleamine 2,3 dioxygenase (IDO) activity during dendritic-cell maturation. *Blood* **106**, 2375–2381.
57. Yasui, H., Takai, K., Yoshida, R. *et al.* (1986) Interferon enhances tryptophan metabolism by inducing pulmonary indoleamine 2,3-dioxygenase: its possible occurrence in cancer patients. *Proceedings of the National Academy of Sciences USA* **83**, 6622–6626.
58. Chon, S.Y., Hassanain, H.H. and Gupta, S.L. (1996) Cooperative role of interferon regulatory factor 1 and p91 (STAT1) response elements in interferon-γ -inducible expression of human indoleamine 2,3-dioxygenase gene. *Journal of Biological Chemistry* **271**, 17247–17252.
59. Barton, B.E. (2006) STAT3: a potential therapeutic target in dendritic cells for the induction of transplant tolerance. *Expert Opinion on Therapeutic Targets* **10**, 459–470.
60. Orabona, C., Puccetti, P., Vacca, C. *et al.* (2006) Towards the identification of a tolerogenic signature in IDO-competent dendritic cells. *Blood* **107**, 2846–2854.
61. Muller, A.J., DuHadaway, J.B., Donover, P.S. *et al.* (2005) Inhibition of indoleamine 2,3-dioxygenase, an immunoregulatory target of the cancer suppression gene *Bin1*, potentiates cancer chemotherapy. *Nature Medicine* **11**, 312–319.
62. Orabona, C., Tomasello, E., Fallarino, F. *et al.* (2005) Enhanced tryptophan catabolism in the absence of the molecular adapter DAP12. *European Journal of Immunolology* **35**, 3111–3118.
63. Manlapat, A.K., Kahler, D.J., Chandler, P.R. *et al.* (2007) Cell-autonomous control of interferon type I expression by indoleamine 2,3-dioxygenase in regulatory $CD19^+$ dendritic cells. *European Journal of Immunolology* **37**, 1064–1071.
64. Robinson, C.M., Hale, P.T. and Carlin, J.M. (2006) NF-κB activation contributes to indoleamine dioxygenase transcriptional synergy induced by IFN-γ and tumor necrosis factor-α. *Cytokine* **35**, 53–61.
65. Fujigaki, H., Saito, K., Fujigaki, S. *et al.* (2006) The signal transducer and activator of transcription 1a and interferon regulatory factor 1 are not essential for the induction of indoleamine 2,3-dioxygenase by lipopolysaccharide: involvement of p38 mitogen-activated

protein kinase and nuclear factor-κB pathways, and synergistic effect of several proinflammatory cytokines. *Journal of Biochemistry* **139**, 655–662.
66. Yuan, W., Collado-Hidalgo, A., Yufit, T. *et al.* (1998) Modulation of cellular tryptophan metabolism in human fibroblasts by transforming growth factor-b: selective inhibition of indoleamine 2,3-dioxygenase and tryptophanyl-tRNA synthetase gene expression. *Journal of Cellular Physiology* **177**, 174–186.
67. Oertelt-Prigione, S., Mao, T.K., Selmi, C. *et al.* (2008) Impaired indoleamine 2,3-dioxygenase production contributes to the development of autoimmunity in primary biliary cirrhosis. *Autoimmunity* **41**, 92–99.
68. Ueno, A., Cho, S., Cheng, L. *et al.* (2007) Transient upregulation of indoleamine 2,3-dioxygenase in dendritic cells by human chorionic gonadotropin downregulates autoimmune diabetes. *Diabetes* **56**, 1686–1693.
69. Zhu, W.H., Lu, C.Z., Huang, Y.M. *et al.* (2007) A putative mechanism on remission of multiple sclerosis during pregnancy: estrogen-induced indoleamine 2,3-dioxygenase by dendritic cells. *Multiple Sclerosis* **13**, 33–40.
70. von Bergwelt-Baildon, M.S., Popov, A., Saric, T. *et al.* (2006) CD25 and indoleamine 2,3-dioxygenase are up-regulated by prostaglandin E2 and expressed by tumor-associated dendritic cells in vivo: additional mechanisms of T-cell inhibition. *Blood* **108**, 228–237.
71. Fallarino, F. and Puccetti, P. (2006) Toll-like receptor 9-mediated induction of the immunosuppressive pathway of tryptophan catabolism. *European Journal of Immunolology* **36**, 8–11.
72. Mellor, A.L., Baban, B., Chandler, P.R. *et al.* (2005) Cutting Edge: CpG oligonucleotides induce splenic $CD19^{+}$ dendritic cells to acquire potent indoleamine 2,3-dioxygenase-dependent T cell regulatory functions via IFN type 1 signaling. *Journal of Immunology* **175**, 5601–5605.
73. Wingender, G., Garbi, N., Schumak, B. *et al.* (2006) Systemic application of CpG-rich DNA suppresses adaptive T cell immunity via induction of IDO. *European Journal of Immunolology* **36**, 12–20.
74. Tsujimura, H., Tamura, T., Kong, H.J. *et al.* (2004) Toll-like receptor 9 signaling activates NF-κB through IFN regulatory factor-8/IFN consensus sequence binding protein in dendritic cells. *Journal of Immunology* **172**, 6820–6827.
75. Romani, L., Bistoni, F., Perruccio, K. *et al.* (2006) Thymosin a1 activates dendritic cell tryptophan catabolism and establishes a regulatory environment for balance of inflammation and tolerance. *Blood* **108**, 2265–2274.
76. Jung, I.D., Lee, C.M., Jeong, Y.I. *et al.* (2007) Differential regulation of indoleamine 2,3-dioxygenase by lipopolysaccharide and interferon-γ in murine bone marrow derived dendritic cells. *FEBS Letters* **581**, 1449–1456.
77. Suh, H.S., Zhao, M.L., Rivieccio, M. *et al.* (2007) Astrocyte indoleamine 2,3-dioxygenase is induced by the TLR3 ligand poly(I:C): mechanism of induction and role in antiviral response. *Journal of Virology* **81**, 9838–9850.
78. Furset, G., Floisand, Y. and Sioud, M. (2008) Impaired expression of indoleamine 2,3-dioxygenase in monocyte-derived dendritic cells in response to Toll-like receptor-7/8 ligands. *Immunology* **123**, 263–271.
79. Orabona, C., Belladonna, M.L., Vacca, C. *et al.* (2005) Cutting edge: silencing suppressor of cytokine signaling 3 expression in dendritic cells turns CD28-Ig from immune adjuvant to suppressant. *Journal of Immunology* **174**, 6582–6586.
80. Orabona, C., Grohmann, U., Belladonna, M.L. *et al.* (2004) CD28 induces immunostimulatory signals in dendritic cells via CD80 and CD86. *Nature Immunology* **5**, 1134–1142.
81. Costa-Pereira, A.P., Tininini, S., Strobl, B. *et al.* (2002) Mutational switch of an IL-6 response to an interferon-γ-like response. *Proceedings of the National Academy of Sciences USA* **99**, 8043–8047.
82. Fallarino, F., Asselin-Paturel, C., Vacca, C. *et al.* (2004) Murine plasmacytoid dendritic cells initiate the immunosuppressive pathway of tryptophan catabolism in response to CD200 receptor engagement. *Journal of Immunology* **173**, 3748–3754.
83. Gorczynski, R.M., Chen, Z., Yu, K. *et al.* (2001) CD200 immunoadhesin suppresses collagen-induced arthritis in mice. *Clinical Immunology* **101**, 328–334.

84. Lawrence, T., Bebien, M., Liu, G.Y. *et al.* (2005) IKKα limits macrophage NF-κB activation and contributes to the resolution of inflammation. *Nature* **434**, 1138–1143.
85. Kinoshita, D., Hirota, F., Kaisho, T. *et al.* (2006) Essential role of IκB kinase a in thymic organogenesis required for the establishment of self-tolerance. *Journal of Immunology* **176**, 3995–4002.
86. Bonizzi, G. and Karin, M. (2004) The two NF-κB activation pathways and their role in innate and adaptive immunity. *Trends in Immunology* **25**, 280–288.
87. Grohmann, U., Bianchi, R., Orabona, C. *et al.* (2003) Functional plasticity of dendritic cell subsets as mediated by CD40 versus B7 activation. *Journal of Immunology* **171**, 2581–2587.
88. Vacca, C., Fallarino, F., Perruccio, K. *et al.* (2005) CD40 ligation prevents onset of tolerogenic properties in human dendritic cells treated with CTLA-4-Ig. *Microbes and Infection* **7**, 1040–1048.
89. Tas, S.W., Vervoordeldonk, M.J., Hajji, N. *et al.* (2007) Non-canonical NF-κB signaling in dendritic cells is required for indoleamine 2,3-dioxygenase (IDO) induction and immune regulation. *Blood* **110**, 1540–1549.
90. Guillonneau, C., Hill, M., Hubert, F.X. *et al.* (2007) CD40Ig treatment results in allograft acceptance mediated by CD8CD45RC T cells, IFN-γ, and indoleamine 2,3-dioxygenase. *Journal of Clinical Investigation* **117**, 1096–1106.
91. Bettelli, E., Sullivan, B., Szabo, S.J. *et al.* (2004) Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis. *Journal of Experimental Medicine* **200**, 79–87.
92. Willenborg, D.O., Fordham, S.A., Staykova, M.A. *et al.* (1999) IFN-γ is critical to the control of murine autoimmune encephalomyelitis and regulates both in the periphery and in the target tissue: a possible role for nitric oxide. *Journal of Immunology* **163**, 5278–5286.
93. Murphy, C.A., Langrish, C.L., Chen, Y. *et al.* (2003) Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *Journal of Experimental Medicine* **198**, 1951–1957.
94. Langrish, C.L., Chen, Y., Blumenschein, W.M. *et al.* (2005) IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *Journal of Experimental Medicine* **201**, 233–240.
95. Romani, L. and Puccetti, P. (2007) Controlling pathogenic inflammation to fungi. *Expert Review of Anti-Infective Therapy* **5**, 1007–1017.
96. De Luca, A., Montagnoli, C., Zelante, T. *et al.* (2007) Functional yet balanced reactivity to *Candida albicans* requires TRIF, MyD88, and IDO-dependent inhibition of *Rorc*. *Journal of Immunology* **179**, 5999–6008.
97. Hayashi, T., Beck, L., Rossetto, C. *et al.* (2004) Inhibition of experimental asthma by indoleamine 2,3-dioxygenase. *Journal of Clinical Investigation* **114**, 270–279.
98. Gurtner, G.J., Newberry, R.D., Schloemann, S.R. *et al.* (2003) Inhibition of indoleamine 2,3-dioxygenase augments trinitrobenzene sulfonic acid colitis in mice. *Gastroenterology* **125**, 1762–1773.
99. Bach, J.F. (1994) Insulin-dependent diabetes mellitus as an autoimmune disease. *Endocrine Reviews* **15**, 516–542.
100. Bertera, S., Alexander, A.M., Crawford, M.L. *et al.* (2004) Gene combination transfer to block autoimmune damage in transplanted islets of Langerhans. *Experimental Diabesity Research* **5**, 201–210.
101. Sakurai, K., Zou, J.P., Tschetter, J.R. *et al.* (2002) Effect of indoleamine 2,3-dioxygenase on induction of experimental autoimmune encephalomyelitis. *Journal of Neuroimmunology*. **129**, 186–196.
102. Xiao, B.G., Wu, X.C., Yang, J.S. *et al.* (2004) Therapeutic potential of IFN-γ-modified dendritic cells in acute and chronic experimental allergic encephalomyelitis. *International Immunology* **16**, 13–22.
103. Schroecksnadel, K., Kaser, S., Ledochowski, M. *et al.* (2003) Increased degradation of tryptophan in blood of patients with rheumatoid arthritis. *Journal of Rheumatology* **30**, 1935–1939.

104. Sawitzki, B., Kingsley, C.I., Oliveira, V. *et al.* (2005) IFN-γ production by alloantigen-reactive regulatory T cells is important for their regulatory function in vivo. *Journal of Experimental Medicine* **201**, 1925–1935.
105. Widner, B., Sepp, N., Kowald, E. *et al.* (1999) Degradation of tryptophan in patients with systemic lupus erythematosus. *Advances in Experimental Medicine and Biology* **467**, 571–577.
106. Pertovaara, M., Raitala, A., Uusitalo, H. *et al.* (2005) Mechanisms dependent on tryptophan catabolism regulate immune responses in primary Sjogren's syndrome. *Clinical and Experimental Immunology* **142**, 155–161.
107. Zhu, L., Ji, F., Wang, Y. *et al.* (2006) Synovial autoreactive T cells in rheumatoid arthritis resist IDO-mediated inhibition. *Journal of Immunology* **177**, 8226–8233.
108. Seo, S.K., Choi, J.H., Kim, Y.H. *et al.* (2004) 4-1BB-mediated immunotherapy of rheumatoid arthritis. *Nature Medicine* **10**, 1088–1094.
109. Choi, B.K., Asai, T., Vinay, D.S. *et al.* (2006) 4-1BB-mediated amelioration of experimental autoimmune uveoretinitis is caused by indoleamine 2, 3-dioxygenase-dependent mechanisms. *Cytokine* **34**, 233–242.
110. Genovese, M.C., Becker, J.C., Schiff, M. *et al.* (2005) Abatacept for rheumatoid arthritis refractory to tumor necrosis factor a inhibition. *New England Journal of Medicine* **353**, 1114–1123.
111. Alexander, A.M., Crawford, M., Bertera, S. *et al.* (2002) Indoleamine 2,3-dioxygenase expression in transplanted NOD Islets prolongs graft survival after adoptive transfer of diabetogenic splenocytes. *Diabetes* **51**, 356–365.

Abstract

In the complex control of tolerogenic responses, the enzyme indoleamine 2,3-dioxygenase (IDO) represents a crucial participant, with multiple functions occurring through multiple mechanisms at multiple levels. Expressed by different subsets of dendritic cells, IDO regulates the first- and rate-limiting step in L-tryptophan catabolism, along a metabolic pathway eventually leading to L-tryptophan deprivation and accumulation of tolerogenic catabolites. In one such local microenvironment where IDO is expressed by regulatory dendritic cells, effector T cells are negatively modulated and regulatory T cells (Tregs) use IDO as an effector mechanism. Modulation of IDO-encoding gene transcription occurs through soluble (type I/II interferons, hormones and Toll-like receptor ligands) and membrane-anchored molecules (cytotoxic T lymphocyte antigen 4 (CTLA-4) and glucocorticoid-induced tumour necrosis factor receptor ligand (GITRL)). An increase in IDO activity is also associated with *de novo* generation of Tregs (whereby Tregs induce Tregs), and this will suppress T helper (Th) 1/Th17 development and, subsequently, autoimmune responses.

Key words: IDO; tolerance; autoimmunity; Treg; tryptophan; kynurenines; DC; *INDO*

PART II

Stress Responses that Break Immune Silence

The Epigenetics of Autoimmune Diseases Edited by Moncef Zouali
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7

Chromatin modifications, oxidative stress and nucleosome autoantibodies

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7.1 Introduction

Systemic lupus erythematosus (SLE) is a rheumatic autoimmune disease affecting over 1 million people in the USA, mostly women (the female/male ratio is 8 : 1 among patients). SLE is a systemic inflammatory disease with skin, renal, joint, cardiovascular and nervous manifestations. The aetiology of the disease is still unknown, although it is believed to be induced by a combination of genetic and environmental factors. The disease is characterized by a broad autoimmune response and the production of numerous autoantibodies (autoAbs) of diverse specificities. It is thought that the resulting immune complexes activate the complement system and deposit and accumulate in kidney glomeruli, joints and vessel walls, causing inflammation and tissue damage in the form of a type III hypersensitivity reaction, which manifests as glomerulonephritis, arthritis and general vasculitis, respectively. SLE is therefore classified as an immune-complex-mediated disease.

Among the targets recognized by autoAbs is the nucleosome, a major autoantigen (autoAg) in SLE. Although anti-double-stranded (ds) DNA autoAbs represent a disease marker in SLE, anti-nucleosome autoAbs (also called nucleosome-restricted autoAbs because they specifically recognize conformational nucleosomal epitopes) represent an antibody population of increasing interest in SLE.

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7.2 Nucleosome and SLE

The genome of each eukaryotic cell is encoded in the DNA and is mainly located in the nucleus. The DNA is condensed in association with basic proteins (histones) to form chromatin. The smallest unit of chromatin is called the nucleosome. The nucleosome core particle consists of a 146-base-pair (bp) DNA section which is wrapped in 1.65 turns around a protein core, the so-called histone octamer [1]. An extra linker histone H1 is bound to the DNA outside the core particle to form a mono-nucleosome containing 180 bp of DNA. This facilitates the formation of the 30 nm fibre in which the nucleosomes coil up in a helical structure to form the chromatin thread. An additional 20–60 bp of linker DNA connect neighbouring nucleosomes and give the 'beads on a string' appearance of the chromatin (Figure 7.1).

The histone octamer is composed of four histones, namely H2A, H2B, H3 and H4, which are highly conserved through evolution. One nucleosome core particle consists of two H2A–H2B dimers and a H3–H4 tetramer, which form a globular structure together. The N-terminal tails of the histone proteins stick out of this structure and are subjected to a variety of post-translational modifications such as acetylation or phosphorylation. Beyond their structural functions, histones are involved in the regulation of genetic information. Different histone modifications participate in transcription, DNA-repair or replication processes. The nucleosomal structure additionally allows or prevents DNA–protein interactions by exposing or hiding DNA regions.

In healthy organisms, nucleosomes are usually not released into the circulation. Nevertheless, circulating nucleosomes are detected in some pathological situations, especially in SLE [2] where they are known as major autoAgs, able to elicit the production of pathogenic autoAbs. Indeed, autoAbs against nucleosomal components (i.e. dsDNA and histones) as well as nucleosome-specific autoAbs are detected in SLE

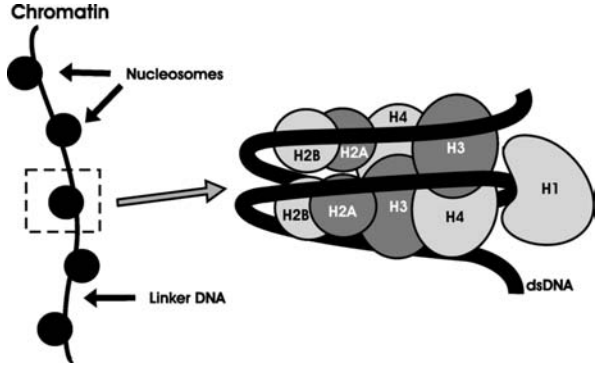

Figure 7.1 Schematic representation of the nucleosome. Chromatin is organized in nucleosomes, the fundamental DNA packing units. Each nucleosome (on the right) is composed of 180 bp of DNA, one histone H1 and two copies each of histones H2A, H2B, H3 and H4. Individual nucleosomes are connected by linker DNA.

patients. Interestingly, nucleosome-specific autoAbs show no or only a very low reactivity against native DNA or individual histones [3]. These autoAbs emerge before anti-DNA or anti-histone antibodies and appear long before the onset of clinical symptoms. This suggests that nucleosomes give also rise to anti-histone and anti-dsDNA antibodies, the so-called golden marker of SLE. The early anti-nucleosome response is multiclonal as anti-nucleosome antibodies recognize diverse quaternary epitopes on nucleosomes in lupus-prone mice [4]. During the progression of the autoimmune response, antibodies reactive against the single nucleosomal components (DNA and histones) evolve. The evolution includes an isotype switch of antibodies and the dominance of IgGs subclasses as well as a rapid increase in autoAb titre, characterizing a T-cell-dependent mechanism. In fact, nucleosome-specific T helper (Th) cells were found in patients with SLE [5]. Pathogenic B lymphocytes are then able to produce the above-described autoAbs with the help of these Th cells. Several Th-cell epitopes have been described in the four core histones and additionally on histone H1. Interestingly, some Th-cell epitopes overlap with epitopes targeted by autoAbs.

Nucleosome-specific autoAbs are only detected in a very restricted set of connective tissue diseases like SLE, systemic sclerosis and mixed connective tissue disease (see [6] for review; Table 7.1). They have been suggested to be a sensitive and specific marker for SLE. Amoura *et al.* showed that titres of nucleosome-specific antibodies of the IgG3 isotype correlate with the disease activity in SLE [7]. Indeed, anti-nucleosome autoAbs are believed to be pathogenic in the form of immune complexes upon deposition in tissues, leading to complement activation and inflammation. Anti-nucleosomal antibodies, free nucleosomes and resulting immune complexes are suggested to play a major role in lupus nephritis. It has been shown that nucleosome–anti-nucleosome complexes bind to the glomerular basement membrane in the kidney. Binding occurs to some extent via the positively charged N-terminal histone tails to heparan sulphate. Interestingly, monoclonal anti-histone antibodies are able to target the cationic histone sequences, depending on the recognized epitopes, and thus prevent binding [8].

Nucleosome-containing immune complexes are additionally capable to activate B lymphocytes as well as dendritic cells, at least to some extent via Toll-like receptor (TLR) 9 (see [9] for review). Furthermore, free nucleosomes are able to activate

Table 7.1 IgGs anti-nucleosome antibody frequency.

	Frequency (%)
Controls	0–5
SLE	31–100
MCTD	3–70
SCL	0–67
SS	0–10

SLE, systemic lupus erythematosus; MCTD, mixed connective tissue disease; SCL, scleroderma (systemic sclerosis); SS, Sjögren's syndrome (see [6] for references). Several ethnic origins are included.

immune cells, such as dendritic cells and neutrophils, in the absence of specific autoAbs [10,11]. The activation processes can result in an amplification loop through the release of interleukin (IL) 8, a known chemoattractant for additional neutrophils. As a consequence, granule contents and reactive oxygen species (ROS) are set free by inflammatory cells in an uncontrolled manner which in turn may lead to an increase in tissue damage.

The inflammatory reactions described above represent a hallmark of SLE. Several factors including genetic and environmental aspects may trigger the disease. Genetic dependencies were analysed by comparing SLE in monozygotic and dizygotic twins [12]: it was shown that 24% of the monozygotic twin pairs were concordant for SLE compared to 2% of the dizygotic twins, suggesting an influence of genetic factors in the disease. It is of note that some chromosomal regions carry SLE susceptibility genes, such as major histocompatibility complex (MHC) class II, Fcγ receptor and cytokine genes (see [13] for review). Lupus has also been associated with exposure to environmental agents like silica, aromatic amines and tobacco. Furthermore, virus infections have been linked to the development of SLE (for review see [14]). The association between viral infections, especially by Epstein–Barr virus, and SLE might contingent upon a cross-reactivity of virus-specific antibodies with SLE auto-Ags [15]. A special and reversible form of lupus is triggered by several drugs such as procainamide, hydralazine and others.

Circulating nucleosomes in the sera of lupus patients are thought to be a product of apoptosis. An impaired clearance of apoptotic cells as well as an augmented apoptosis rate might explain the presence of circulating nucleosomes in SLE patients. In fact, several studies have been published describing defective phagocytosis. It was demonstrated that monocyte-derived macrophages as well as tingible body macrophages from SLE patients are affected in their phagocytic capacity. High amounts of non-ingested apoptotic cells were observed in germinal centres and cell-culture experiments, and additionally only few macrophages contained engulfed cell debris [16,17]. Different *in vitro* studies showed that immune cells of SLE patients, like lymphocytes and neutrophils, seem to be more prone to apoptosis compared to those of healthy controls or of patients with other chronic inflammatory diseases [18,19].

Nucleosomes are generated from chromatin by nucleases during apoptosis. Together with other nuclear components they cluster on the surface of apoptotic blebs [20]. If apoptotic cells are not cleared properly they undergo secondary necrosis and release their modified noxious contents. There are several effector molecules suggested to be involved in the clearance of apoptotic cells and degraded chromatin, once it is released into the circulation. Among them are serum nucleases, such as deoxyribonuclease 1 (DNase I), the activity of which is decreased during active stages of SLE [21]. Its ability to degrade chromatin from necrotic cells is facilitated and increased in the presence of the complement component C1q [22], which is known to promote the clearance of apoptotic cells by binding to the surface blebs of apoptotic cells [23,24]. C1q circulating levels are decreased in lupus patients with active disease, suggesting that a deficiency, inherited or acquired, predisposes to the development of an SLE-like disease [25]. Indeed, 90% of individuals with a complete absence of this molecule develop SLE.

Proteins of the acute phase response, the so-called pentraxins, serve additionally as opsonins for apoptotic cells and are known to enhance their phagocytosis [26–28]. A low C-reactive protein response has been observed in SLE patients during the active phase of the disease [29]. Additionally, C-reactive protein and its relative serum amyloid P are also able to bind directly to nucleosomes [30,31]. Low serum levels of these molecules might therefore favour the development of an anti-nucleosomal immune reaction.

7.3 Epigenetics and SLE

The question remains as to why the nucleosome becomes immunogenic and why it occurs only in a patient subpopulation. This raises the possibility that epigenetic factors are involved in triggering anti-nucleosome autoimmunity. Epigenetics covers chromatin (DNA and associated proteins) modifications that do not entail a change in the DNA sequence, resulting in changes in gene expression. Epigenetic information is heritable during cell division. The major chromatin modifications consist of DNA methylation and post-translational modifications of histones, especially at the N-terminal tails. DNA methylation is mediated by DNA methyltransferases and commonly occurs at cytosines in CpG DNA motifs. In particular, DNA methylation in CpG islands, which are often located in promoter regions, results in transcriptional repression. Post-translational histone modifications control chromatin remodelling and the access to the underlying DNA, thereby regulating gene expression. Numerous histone modifications have been reported (e.g. acetylation, phosphorylation, methylation). They are controlled by specific enzymes and associated with diverse cellular processes. For example, acetylation of lysines in histone tails is a reversible process which causes the unwinding of chromatin structure and therefore allows transcription factors to access promoter sites. Histone acetylation is regulated by acetyltransferases (which promote acetylation) and deacetylases (which promote deacetylation). The combination of all histone modifications forms the 'histone code', extending the information potential of the genetic code [32]. Importantly, DNA methylation and histone modifications are mechanistically linked, implying that changes in DNA methylation are associated with histone modification changes. The chromatin state is fundamental for gene expression, and chromatin remodelling factors can modify the balance between euchromatin (active chromatin with lightly packed DNA) and heterochromatin (inactive chromatin with tightly packed DNA) by acting as main regulators of gene expression. Besides a role in gene expression and chromatin organization, epigenetics indirectly comprises the biochemical and structural nucleosome modifications that might control nucleosome immunogenicity. Studies on monozygotic twins have highlighted the importance of epigenetic factors in SLE development. The highest concordance rate for SLE in twins is about 60% [12,33], supporting the involvement of non-germline factors in SLE pathogenesis. Thus, as mentioned above, environmental factors (including infectious agents, chemicals, medication, diet, lifestyle) contribute to lupus pathogenesis (see [34] for review).

Such environmental factors influence the epigenetic profile of each individual [35], which might partly explain how the environment modulates genetic predisposition to SLE. Importantly, epigenetic alterations accumulate during an individual's lifetime [36]. Thus, the analysis of a large cohort of monozygotic twins revealed that twins are epigenetically indistinguishable during the early years of life, but exhibit remarkable epigenetic differences with age. This result explains why genetically identical individuals exposed to different environments do not have the same disease susceptibility or why they do not show the disease at the same age.

7.4 Oxidative stress in SLE: definition and mechanisms

Oxygen metabolism is essential for life but is potentially threatening to cells due to the formation of ROS and their conversion into reactive nitrogen species (RNS). Oxidative stress is caused by an imbalance between the production of oxidants and the antioxidant capacity. It is thus a critical condition with overproduction of oxidants and/or decreased ability to detoxify those oxidants. Although some ROS/RNS are involved in important processes such as cell signalling or immune responses, they can also damage cell components, including lipids, proteins and DNA. For example, nuclear factor κB (NF-κB) activation has been suggested to be regulated by the levels of oxidants inside cells. Disturbance in the normal redox state leads to the production of toxic ROS, which include free radicals and peroxides, and toxic RNS. Among them, the superoxide anion ($^{\bullet}O_2^-$) is rather unreactive but can be converted in more aggressive species, whereas hydroxyl radical ($^{\bullet}OH$) is extremely reactive. Hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), peroxynitrite ($OONO^-$) and nitric oxide ($NO^{\bullet}$) are other key players in the redox state. The enzymatic and non-enzymatic mechanisms leading to ROS/RNS production and elimination are rather complex (see [37] for detailed review). For example, the most important source of reactive oxygen in aerobic organisms is the generation of side products of the oxidative energy metabolism occurring in mitochondria during normal oxidative respiration. This process leads to ATP production and is named oxidative phosphorylation. Besides the mitochondrial electron-transport chain, a series of oxidases are also involved in the generation of the superoxide anion. Superoxide dismutase converts the superoxide anion into hydrogen peroxide which is then converted into water by catalase or glutathione peroxidase. Nitric oxide is produced from arginine by the nitric oxide synthases and can then for example be converted into peroxynitrite upon reaction with the superoxide anion. The redox network is also composed of non-enzymic components such as transition metals, which mediate similar reactions.

Oxidative stress has been suggested to be involved in the development of some diseases, such as neurodegenerative diseases or cardiovascular diseases. Thus in Alzheimer's disease hydrogen peroxide mediates amyloid β protein toxicity [38]. In atherosclerosis, oxidized low-density lipoproteins (involved in plaque formation) activate monocytes and macrophages, leading to hydrogen peroxide generation [39]. Oxidative stress has also been implicated in autoimmune disease, such as rheumatoid

arthritis and diabetes. It has also been analysed in the context of SLE. Increased oxidative stress levels have been reported in SLE patients [40]. Interestingly, vascular disease and atherosclerosis are common clinical manifestations in SLE. This observation, however, was not confirmed in a recent study involving SLE patients with less severe disease [41]. Nevertheless, sera from SLE patients have been shown to contain increased levels of protein oxidation and patients with aggressive disease and with high anti-dsDNA levels exhibited the highest oxidation levels [42]. Moreover, mice deficient in a transcription factor responsible for controlling the expression of detoxification and antioxidant genes develop a lupus-like disease [43].

Several mechanisms may explain oxidative stress induction and its involvement in SLE. As mentioned above, SLE develops when a combination of genetic and environmental factors are encountered. Particularly, bacterial infections may represent a triggering factor for SLE. Upon bacterial infection, neutrophils and macrophages produce and partly release ROS which are normally involved in the killing of bacteria, a process named respiratory burst. The release of ROS, especially upon strong activation, may cause damage to surrounding tissues. Similarly, strong ROS generation might alter intracellular material and lead to epigenetic modifications. Such responses are also associated with cytokine secretion, such as the chemoattractant IL-8 by neutrophils, favouring the development of inflammation. We should keep in mind that SLE is an inflammatory disease and that neutrophils are the first cells recruited at sites of inflammation. High ROS release might particularly occur during frustrated phagocytosis by neutrophils, which occurs when phagocytic cells bind to a surface that cannot be phagocytosed. This mechanism would explain some tissue lesions observed in SLE when neutrophils recognize immune complexes or autoAgs bound to fixed surfaces, especially on the glomerular basement membrane in the kidneys. Interestingly, we have shown that circulating nucleosomes induce neutrophil activation without requiring immune-complex formation [11], particularly leading to oxidative burst in neutrophils (V. Rönnefarth and P. Decker, unpublished work). Nucleosomes are present in glomerular deposits in human lupus nephritis [44], suggesting that circulating nucleosomes deposit and are trapped on the glomerular basement membrane. It can therefore be hypothesized that planted nucleosomes might also activate neutrophils *in situ*, leading to ROS release, inflammation and kidney damage. Similarly, nucleosomes have been detected in the epidermal basement membrane of the skin of SLE patients [45], suggesting that nucleosome-mediated neutrophil activation and ROS release *in situ* may occur at sites other than the glomeruli.

As mentioned above, lupus patients have an increased risk of atherosclerosis, and oxidative stress is believed to contribute to this manifestation. Recently, plasmas from SLE patients have been shown to contain antibodies directed against high-density lipoproteins that inhibit paraoxonase, an antioxidant enzyme present in high-density lipoproteins [46]. Likewise, anticardiolipin antibodies seem to induce nitrite oxide and superoxide production, resulting in increased plasma levels of peroxynitrite [47].

Lupus patients are known to be photosensitive and skin lesions are a common manifestation in SLE patients. Thus, keratinocytes have been suggested to be involved in the pathogenesis of lupus photosensitivity. Interestingly, human primary

keratinocytes produce and accumulate ROS upon exposure to ultraviolet light [48], which may result in damage of cellular autoAgs and may cause structure alterations, revealing cryptic or neo-epitopes. Moreover, lupus keratinocytes undergo apoptosis upon exposure to ultraviolet light which, in a pro-inflammatory environment, may lead to immune responses against self-antigen exposed on apoptotic keratinocytes. Particularly, ultraviolet B radiation induces autoAg relocalization and apoptosis in human keratinocytes [48]. Intermediate and high doses of ultraviolet B radiation were shown to induce pro-inflammatory apoptosis and necrosis, respectively, where the production of inflammatory cytokines is accompanied by exposure or release of autoAg [49]. Consistently, it was shown that nucleosomes are exposed at the surface of apoptotic cells [50].

ROS are known to induce DNA damages, especially DNA breaks. In response to this genotoxic injury, the enzyme poly(ADP-ribose) polymerase (PARP) 1 is activated and synthesizes from β-NAD^+ a poly(ADP-ribose) homopolymer, a reaction with an energetic cost because β-NAD^+ is necessary for ATP synthesis. This polymer is covalently attached to diverse acceptor proteins, such as histones. This post-translational modification alters chromatin structure and contributes to the histone code (see [51] for review). Thus, induction of ROS leads to this particular epigenetic alteration. Due to the energy cost caused by PARP-1 activation, this enzyme has been suspected to control the commutation of the cell-death pathway. Indeed, cell death can switch from apoptosis to necrosis, which occurs when the ATP pool is depleted. Thus, upon ROS generation PARP is hyper-activated, leading to ATP consumption and consequently causing necrosis and potentially inflammation upon release of cell content and potentially altered autoAg. Moreover, PARP-1 has been suggested to modulate inflammation by regulating some genes, such as inducible nitric oxide synthase (iNOS). PARP-1 is indeed required for NF-κB activation [52] and NF-κB is involved in iNOS transcription, leading to nitric oxide and then peroxynitrite production and DNA damage. Thus PARP is activated by ROS and generates oxidants. Interestingly, a locus containing the *PARP* gene has been suggested to be associated with SLE development, although this is still a matter of debate. Moreover, anti-PARP autoAbs are detected in SLE patients [53,54], some of them being potentially pathogenic [55,56]. It should be noted that DNA breaks are induced by several genotoxic stresses (e.g. γ-radiation, monofunctional alkylating agents) and not only by ROS, leading to PARP activation and histone modification. This clearly shows that environmental factors lead to epigenetic alteration with ROS being involved at different steps. Moreover, DNA breaks themselves could be considered as an epigenetic alteration.

As mentioned above, inflammatory reactions in response to bacterial infections are associated with ROS production. Upon activation of neutrophils or macrophages iNOS is activated and releases nitric oxide in large quantities, which is in turn converted to peroxynitrite and is responsible for DNA damage. PARP is then activated, leading to depletion in NAD^+ and ATP pools, and necrosis of target cells in extreme cases. This situation reflects how environmental factors may affect the oxidative status and the consequences on epigenetic alterations of self proteins, inflammation and the potential autoimmune response in SLE.

Increased rates of apoptosis have been suggested to favour SLE development whereas necrosis, which is accompanied by the release of cell content, might favour inflammation. Indeed, apoptosis might generate more immunogenic autoAgs and lead indirectly to increased circulating autoAgs, thus partly explaining how peripheral tolerance is broken. Interestingly, the mode of cell death depends on the cellular energy state [57] and the strength of oxidative stress may indirectly control the mode of cell death [58]. Then, ATP is required for apoptosis whereas depletion of ATP pools leads to necrosis. Moreover, upon PARP activation oxidative stress might lead to ATP depletion and necrosis. Thus, a fine balance or the combination of both modes of cell death might be crucial in lupus development.

7.5 Oxidative stress, epigenetic alterations and nucleosome immunogenicity

Several epigenetic alterations have been suggested to favour the development of anti-chromatin autoAbs. Some of them may be directly or indirectly modulated by oxidative stress. In this section, we will describe some modifications in the context of the broad anti-nucleosome autoAb family (anti-dsDNA, anti-histone, nucleosome-restricted autoAbs) as some studies were performed before the description of the nucleosome-restricted autoAbs [3] or did not compare the different antibody populations. Thus, normal mice immunized with poly(ADP-ribose) develop antibodies cross-reacting with DNA [59]. Similar autoAbs were also detected in non-immunized lupus mice. Moreover, the level of 8-oxo-deoxyguanosine, a DNA lesion induced by ROS, is significantly higher in the DNA from lupus lymphocytes as compared to normal lymphocytes [60]. In addition, sera from lupus patients were shown to recognize this DNA oxidative damage [61]. Although nitric oxide is not a strong oxidant, it might contribute to SLE pathogenesis. Elevated levels of nitric oxide have been reported in SLE patients [62] and lupus autoAbs have been shown to preferentially bind to nitric oxide-modified DNA as compared to untreated DNA [63]. Moreover, although native DNA is a weak immunogen, ROS-modified DNA is immunogenic and is recognized by anti-dsDNA antibodies isolated from lupus patients [64]. Both antibodies induced by ROS-modified DNA and anti-DNA autoAbs derived from SLE patients showed similar antigenic binding characteristics [65]. Similarly, DNA modified with the potent oxidant peroxynitrite is highly immunogenic and antibodies induced against the modified DNA cross-react with native DNA [66]. Likewise, exposure of DNA to ROS or ultraviolet light increases DNA immunogenicity and induces antibodies recognizing both native and modified DNA in rabbits [67]. These results suggest that self DNA damaged upon oxidative stress might favour the break of peripheral tolerance and the development of anti-dsDNA autoAbs in lupus patients. As far as DNA is concerned, another important epigenetic modification might be associated with an increased immunogenicity. As mentioned above, DNA methylation is a major epigenetic mark. Thus, T lymphocytes from patients with active lupus were shown to exhibit globally

hypomethylated DNA [68]. Interestingly, moderate oxidative stress can induce apoptosis and increased rates of apoptosis are observed in lupus patients. Moreover, apoptotic DNA has been recently shown to exhibit lower methylation than normal or necrotic DNA and to induce a lupus-like disease upon injection in normal mice, including the development of anti-dsDNA autoAbs [69]. Those results suggest that TLR9 (the receptor for unmethylated CpG DNA motifs) might be involved in the recognition of apoptotic self DNA. Nevertheless, DNA is not circulating as a free molecule in SLE patients but in the form of nucleosomes [2]. Thus, circulating nucleosomes released from apoptotic cells upon oxidative stress might contain hypomethylated DNA, rendering them more immunogenic, at least partly via a TLR9-dependent pathway. Accordingly, we have shown that free nucleosomes activate dendritic cells as well as neutrophils [10,11]. DNA methylation in CpG islands often occurs in the promoter and first exon of genes and is associated with transcriptional repression. Thus, these demethylation events might also be associated with some phenotypic changes observed in SLE. Likewise, hypomethylation of regulatory sequences may result in the increased expression of human endogenous retroviruses. Since the components of such viruses exhibit a high similarity with nuclear antigens, they might enhance the production of anti-nuclear antibodies; for example, anti-dsDNA (see [70] for review).

Similar mechanisms might be involved in the induction of nucleosome-restricted autoAbs. Thus, SLE autoAbs bind with a high specificity to ROS-damaged chromatin as compared to native chromatin [71]. Moreover, peroxidation of polyunsaturated fatty acids occurs in lipoproteins and cell membrane phospholipids during oxidative stress. Particularly, low-density lipoproteins are prone to oxidation and they are involved in the development of atherosclerosis. Lipid peroxidation leads to the formation of different products, such as aldehydes, reactive with proteins. Interestingly, mice immunized with an aldehyde-modified self protein develop T cells specific for the modified protein as well as antibodies against the non-modified self protein [72], demonstrating that oxidized self proteins can induce a break of tolerance. Likewise, lipid peroxidation leads to the generation of another reactive aldehyde (4-hydroxy-2-nonenal) that can modify proteins which have been suggested to represent an endogenous triggering antigen for anti-DNA autoAbs in SLE [73].

Several other epigenetic modifications might increase chromatin immunogenicity and favour the production of anti-nucleosome autoAbs in SLE. Some of them might be induced during oxidative stress, especially those associated with apoptosis because oxidative stress induces apoptosis in some conditions. Thus, histone acetylation and phosphorylation have been observed during apoptosis [74,75]. Importantly, apoptosis-induced histone acetylation has been recently reported as being pathogenic in SLE [76]. The reactivity of antibodies from lupus patients and lupus mice was indeed higher with hyperacetylated and apoptotic histones than with normal or nonacetylated histones. Moreover, injection of an acetylated histone peptide to lupus-prone mice accelerated the disease and hyperacetylated nucleosomes were able to induce dendritic cell activation. On the contrary, it is unknown whether histone phosphorylation triggers anti-nucleosome autoAbs. Nevertheless, ROS have been shown to induce the

phosphorylation of histone H3 [77]. Apoptosis-induced histone transglutamination has been reported but it has not been linked to the production of lupus autoAbs. Surprisingly, deubiquitination of histone H2A also occurs during apoptosis whereas autoAbs against ubiquitinated H2A are detected in lupus mice [78]. Finally, the apoptotic DNA cleavage might be considered as an epigenetic modification but it is unclear whether such cleavage renders the chromatin more immunogenic.

ROS have been shown to oxidize individual histones. Particularly, histone H1 was oxidized in response to treatment with hydrogen peroxide, as estimated by the level of protein-bound carbonyl groups [79,80]. Oxidized histones were also observed in histone–DNA complexes. Oxidation of histones also occurs in cells upon oxidative stress. Oxidatively damaged histones are then degraded by the nuclear proteasome, a process depending on poly(ADP-ribosyl)ation of the proteasome. Those results show that epigenetic modifications may be linked. Nevertheless, the effect of the latter modifications on the production of anti-nucleosome autoAbs is not known.

Another consequence of oxidative stress which may lead to the break of peripheral tolerance, anti-nucleosome production and SLE development is the activation of immune cells. ROS have been shown to activate dendritic cells, which in turn can further stimulate antigen-specific T lymphocytes [81]. Consistently, we have shown that lupus dendritic cells spontaneously over-express CD86, a marker of activation [82]. An epigenetic regulation of genes involved in dendritic cell activation may then favour the break of the peripheral tolerance upon oxidative stress.

7.6 Conclusion

Oxidative stress may result in epigenetic alterations and some of them might modulate nucleosome immunogenicity. Since chromatin modifications are involved in the control of gene expression, oxidative stress may not only render chromatin more immunogenic but may also result in the deregulated expression of some genes. Thus, the combination of both events might lead to the induction of anti-nucleosome autoAbs in SLE. It is possible that deregulated genes belong to pro-apoptotic genes and genes involved in immune responses or even to genes controlling chromatin post-translational modifications. Some of those defects may in turn induce additional epigenetic alterations or the production of aggressive oxidants.

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References

1. Luger, K., Mader, A.W., Richmond, R.K. *et al.* (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* **389**, 251–260.
2. Rumore, P.M. and Steinman, C.R. (1990) Endogenous circulating DNA in systemic lupus erythematosus. Occurrence as multimeric complexes bound to histone. *Journal of Clinical Investigation* **86**, 69–74.
3. Chabre, H., Amoura, Z., Piette, J.C. *et al.* (1995) Presence of nucleosome-restricted antibodies in patients with systemic lupus erythematosus. *Arthritis and Rheumatism* **38**, 1485–1491.
4. Jovelin, F., Mostoslavsky, G., Amoura, Z. *et al.* (1998) Early anti-nucleosome autoantibodies from a single MRL + / + mouse: fine specificity, V gene structure and pathogenicity. *European Journal of Immunology* **28**, 3411–3422.
5. Lu, L., Kaliyaperumal, A., Boumpas, D.T. *et al.* (1999) Major peptide autoepitopes for nucleosome-specific T cells of human lupus. *Journal of Clinical Investigation* **104**, 345–355.
6. Decker, P. (2006) Nucleosome autoantibodies. *Clinica Chimica Acta* **366**, 48–60.
7. Amoura, Z., Koutouzov, S., Chabre, H. *et al.* (2000) Presence of antinucleosome autoantibodies in a restricted set of connective tissue diseases: antinucleosome antibodies of the IgG3 subclass are markers of renal pathogenicity in systemic lupus erythematosus. *Arthritis and Rheumatism* **43**, 76–84.
8. van Bruggen, M.C., Walgreen, B., Rijke, T.P. *et al.* (1997) Antigen specificity of anti-nuclear antibodies complexed to nucleosomes determines glomerular basement membrane binding in vivo. *European Journal of Immunology* **27**, 1564–1569.
9. Rifkin, I.R., Leadbetter, E.A., Busconi, L. *et al.* (2005) Toll-like receptors, endogenous ligands, and systemic autoimmune disease. *Immunological Reviews* **204**, 27–42.
10. Decker, P., Singh-Jasuja, H., Haager, S. *et al.* (2005) Nucleosome, the main autoantigen in systemic lupus erythematosus, induces direct dendritic cell activation via a MyD88-independent pathway: consequences on inflammation. *Journal of Immunology* **174**, 3326–3334.
11. Rönnefarth, V.M., Erbacher, A.I., Lamkemeyer, T. *et al.* (2006) TLR2/TLR4-independent neutrophil activation and recruitment upon endocytosis of nucleosomes reveals a new pathway of innate immunity in systemic lupus erythematosus. *Journal of Immunology* **177**, 7740–7749.
12. Deapen, D., Escalante, A., Weinrib, L. *et al.* (1992) A revised estimate of twin concordance in systemic lupus erythematosus. *Arthritis and Rheumatism* **35**, 311–318.
13. Harley, J.B., Kelly, J.A. and Kaufman, K.M. (2006) Unraveling the genetics of systemic lupus erythematosus. *Springer Seminars in Immunopathology* **28**, 119–130.
14. Edwards, C.J. and Cooper, C. (2006) Early environmental exposure and the development of lupus. *Lupus* **15**, 814–819.
15. Incaprera, M., Rindi, L., Bazzichi, A. *et al.* (1998) Potential role of the Epstein-Barr virus in systemic lupus erythematosus autoimmunity. *Clinical and Experimental Rheumatology* **16**, 289–294.
16. Herrmann, M., Voll, R.E., Zoller, O.M. *et al.* (1998) Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus. *Arthritis and Rheumatism* **41**, 1241–1250.
17. Baumann, I., Kolowos, W., Voll, R.E. *et al.* (2002) Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. *Arthritis and Rheumatism* **46**, 191–201.
18. Emlen, W., Niebur, J. and Kadera, R. (1994) Accelerated in vitro apoptosis of lymphocytes from patients with systemic lupus erythematosus. *Journal of Immunology* **152**, 3685–3692.
19. Courtney, P.A., Crockard, A.D., Williamson, K. *et al.* (1999) Increased apoptotic peripheral blood neutrophils in systemic lupus erythematosus: relations with disease activity, antibodies to double stranded DNA, and neutropenia. *Annals of the Rheumatic Diseases* **58**, 309–314.

20. Casciola-Rosen, L.A., Anhalt, G. and Rosen, A. (1994) Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *Journal of Experimental Medicine* **179**, 1317–1330.
21. Chitrabamrung, S., Rubin, R.L. and Tan, E.M. (1981) Serum deoxyribonuclease I and clinical activity in systemic lupus erythematosus. *Rheumatology International* **1**, 55–60.
22. Gaipl, U.S., Beyer, T.D., Heyder, P. *et al.* (2004) Cooperation between C1q and DNase I in the clearance of necrotic cell-derived chromatin. *Arthritis and Rheumatism* **50**, 640–649.
23. Korb, L.C. and Ahearn, J.M. (1997) C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. *Journal of Immunology* **158**, 4525–4528.
24. Navratil, J.S., Watkins, S.C., Wisnieski, J.J. *et al.* (2001) The globular heads of C1q specifically recognize surface blebs of apoptotic vascular endothelial cells. *Journal of Immunology* **166**, 3231–3239.
25. Walport, M.J., Davies, K.A. and Botto, M. (1998) Clq and systemic lupus erythematosus. *Immunobiology* **199**, 265–285.
26. Bijl, M., Horst, G., Bijzet, J. *et al.* (2003) Serum amyloid P component binds to late apoptotic cells and mediates their uptake by monocyte-derived macrophages. *Arthritis and Rheumatism* **48**, 248–254.
27. Gershov, D., Kim, S., Brot, N. *et al.* (2000) C-Reactive protein binds to apoptotic cells, protects the cells from assembly of the terminal complement components, and sustains an antiinflammatory innate immune response: implications for systemic autoimmunity. *Journal of Experimental Medicine* **192**, 1353–1364.
28. Mold, C., Baca, R. and Du Clos, T.W. (2002) Serum amyloid P component and C-reactive protein opsonize apoptotic cells for phagocytosis through Fcgamma receptors. *Journal of Autoimmunity* **19**, 147–154.
29. Russell, A.I., Cunninghame Graham, D.S., Shepherd, C. *et al.* (2004) Polymorphism at the C-reactive protein locus influences gene expression and predisposes to systemic lupus erythematosus. *Human Molecular Genetics* **13**, 137–147.
30. Du Clos, T.W., Zlock, L.T. and Rubin, R.L. (1988) Analysis of the binding of C-reactive protein to histones and chromatin. *Journal of Immunology* **141**, 4266–4270.
31. Pepys, M.B. and Butler, P.J. (1987) Serum amyloid P component is the major calcium-dependent specific DNA binding protein of the serum. *Biochemical and Biophysical Research Communications* **148**, 308–313.
32. Jenuwein, T. and Allis, C.D. (2001) Translating the histone code. *Science* **293**, 1074–1080.
33. Jarvinen, P. and Aho, K. (1994) Twin studies in rheumatic diseases. *Seminars in Arthritis and Rheumatism* **24**, 19–28.
34. Miller, F.W. and Cooper, G.S. (2007) Environmental aspects of lupus. In: *Dubois' Lupus Erythematosus*, 7th edn (Wallace D.J. and Hahn B.H., eds), pp. 21–33. Lippincott Williams and Wilkins, Philadelphia.
35. Bjornsson H.T., Fallin, M.D. and Feinberg, A.P. (2004) An integrated epigenetic and genetic approach to common human disease. *Trends in Genetics* **20**, 350–358.
36. Fraga, M.F., Ballestar, E., Paz, M.F. *et al.* (2005) Epigenetic differences arise during the lifetime of monozygotic twins. *Proceedings of the National Academy of Sciences, USA* **102**, 10604–10609.
37. Droge, W. (2002) Free radicals in the physiological control of cell function. *Physiological Reviews* **82**, 47–95.
38. Behl, C., Davis, J.B., Lesley, R. *et al.* (1994) Hydrogen peroxide mediates amyloid beta protein toxicity. *Cell* **77**, 817–827.
39. Kinscherf, R., Deigner, H.P., Usinger, C. *et al.* (1997) Induction of mitochondrial manganese superoxide dismutase in macrophages by oxidized LDL: its relevance in atherosclerosis of humans and heritable hyperlipidemic rabbits. *FASEB Journal* **11**, 1317–1328.

40. Ames, P.R., Alves, J., Murat, I. *et al.* (1999) Oxidative stress in systemic lupus erythematosus and allied conditions with vascular involvement. *Rheumatology (Oxford)* **38**, 529–534.
41. Avalos, I., Chung, C.P., Oeser, A. *et al.* (2007) Oxidative stress in systemic lupus erythematosus: relationship to disease activity and symptoms. *Lupus* **16**, 195–200.
42. Morgan, P.E., Sturgess, A.D. and Davies, M.J. (2005) Increased levels of serum protein oxidation and correlation with disease activity in systemic lupus erythematosus. *Arthritis and Rheumatism* **52**, 2069–2079.
43. Li, J., Stein, T.D. and Johnson, J.A. (2004) Genetic dissection of systemic autoimmune disease in Nrf2-deficient mice. *Physiological Genomics* **18**, 261–272.
44. van Bruggen, M.C., Kramers, C., Walgreen, B. *et al.* (1997) Nucleosomes and histones are present in glomerular deposits in human lupus nephritis. *Nephrology Dialysis Transplantation* **12**, 57–66.
45. Grootscholten, C., van Bruggen, M.C., van der Pijl, J.W. *et al.* (2003) Deposition of nucleosomal antigens (histones and DNA) in the epidermal basement membrane in human lupus nephritis. *Arthritis and Rheumatism* **48**, 1355–1362.
46. Batuca, J.R., Ames, P.R., Isenberg, D.A. *et al.* (2007) Antibodies toward high-density lipoprotein components inhibit paraoxonase activity in patients with systemic lupus erythematosus. *Annals of the New York Academy of Science* **1108**, 137–146.
47. Alves, J.D. and Grima, B. (2003) Oxidative stress in systemic lupus erythematosus and antiphospholipid syndrome: a gateway to atherosclerosis. *Current Rheumatology Reports* **5**, 383–390.
48. Lawley, W., Doherty, A., Denniss, S. *et al.* (2000) Rapid lupus autoantigen relocalization and reactive, oxygen species accumulation following ultraviolet irradiation of human keratinocytes. *Rheumatology (Oxford)* **39**, 253–261.
49. Caricchio, R., McPhie, L. and Cohen, P.L. (2003) Ultraviolet B radiation-induced cell death: critical role of ultraviolet dose in inflammation and lupus autoantigen redistribution. *Journal of Immunology* **171**, 5778–5786.
50. Radic, M., Marion, T. and Monestier, M. (2004) Nucleosomes are exposed at the cell surface in apoptosis. *Journal of Immunology* **172**, 6692–6700.
51. Decker, P. and Muller, S. (2002) Modulating poly (ADP-ribose) polymerase activity: potential for the prevention and therapy of pathogenic situations involving DNA damage and oxidative stress. *Current Pharmaceutical Biotechnology* **3**, 275–283.
52. Hassa, P.O. and Hottiger, M.O. (1999) A role of poly (ADP-ribose) polymerase in NF-kappaB transcriptional activation. *Biological Chemistry* **380**, 953–959.
53. Yamanaka, H., Willis, E.H., Penning, C.A. *et al.* (1987) Human autoantibodies to poly(adenosine diphosphate-ribose) polymerase. *Journal of Clinical Investigation* **80**, 900–904.
54. Decker, P., Briand, J.P., de Murcia, G. *et al.* (1998) Zinc is an essential cofactor for recognition of the DNA binding domain of poly(ADP-ribose) polymerase by antibodies in autoimmune rheumatic and bowel diseases. *Arthritis and Rheumatism* **41**, 918–926.
55. Yamanaka, H., Willis, E.H. and Carson, D.A. (1989) Human autoantibodies to poly(adenosine diphosphate-ribose) polymerase recognize cross-reactive epitopes associated with the catalytic site of the enzyme. *Journal of Clinical Investigation* **83**, 180–186.
56. Decker, P., Isenberg, D. and Muller, S. (2000) Inhibition of caspase-3-mediated poly(ADP-ribose) polymerase (PARP) apoptotic cleavage by human PARP autoantibodies and effect on cells undergoing apoptosis. *Journal of Biological Chemistry* **275**, 9043–9046.
57. Leist, M., Single, B., Castoldi, A.F. *et al.* (1997) Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *Journal of Experimental Medicine* **185**, 1481–1486.
58. Szabo, C., Zingarelli, B., O'Connor, M. *et al.* (1996) DNA strand breakage, activation of poly (ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity of macrophages and smooth muscle cells exposed to peroxynitrite. *Proceedings of the National Academy of Sciences USA* **93**, 1753–1758.

59. Sibley, J.T., Braun, R.P. and Lee, J.S. (1986) The production of antibodies to DNA in normal mice following immunization with poly(ADP-ribose). *Clinical and Experimental Immunology* **64**, 563–569.
60. Bashir, S., Harris, G., Denman, M.A. *et al.* (1993) Oxidative DNA damage and cellular sensitivity to oxidative stress in human autoimmune diseases. *Annals of the Rheumatic Diseases* **52**, 659–666.
61. Seedhouse, C.H., Margison, G.P., Hendry, J.H. *et al.* (2001) Anti-8-oxo-2′-deoxyguanosine phage antibodies: isolation, characterization, and relationship to disease states. *Biochemical and Biophysical Research, Communications* **280**, 595–604.
62. Belmont, H.M., Levartovsky, D., Goel, A. *et al.* (1997) Increased nitric oxide production accompanied by the up-regulation of inducible nitric oxide synthase in vascular endothelium from patients with systemic lupus erythematosus. *Arthritis and Rheumatism* **40**, 1810–1816.
63. Dixit, K. and Ali, R. (2004) Role of nitric oxide modified DNA in the etiopathogenesis of systemic lupus erythematosus. *Lupus* **13**, 95–100.
64. Alam, K., Ali, A. and Ali, R. (1993) The effect of hydroxyl radical on the antigenicity of native DNA. *FEBS Letters* **319**, 66–70.
65. Alam, K. and Ali, R. (1999) Human anti-DNA autoantibodies and induced antibodies against ROS-modified-DNA show similar antigenic binding characteristics. *Biochemistry and Molecular Biology International* **47**, 881–890.
66. Dixit, K., Moinuddin and Ali, A. (2005) Immunological studies on peroxynitrite modified human DNA. *Life Sciences* **77**, 2626–2642.
67. Cooke, M.S., Mistry, N., Wood, C. *et al.* (1997) Immunogenicity of DNA damaged by reactive oxygen species–implications for anti-DNA antibodies in lupus. *Free Radical Biology and Medicine* **22**, 151–159.
68. Richardson, B., Scheinbart, L., Strahler, J. *et al.* (1990) Evidence for impaired T cell DNA methylation in systemic lupus erythematosus and rheumatoid arthritis. *Arthritis and Rheumatism* **33**, 1665–1673.
69. Wen, Z.K., Xu, W., Xu, L. *et al.* (2007) DNA hypomethylation is crucial for apoptotic DNA to induce systemic lupus erythematosus-like autoimmune disease in SLE-non-susceptible mice. *Rheumatology (Oxford)* **46**, 1796–1803.
70. Januchowski, R., Prokop, J. and Jagodzinski, P.P. (2004) Role of epigenetic DNA alterations in the pathogenesis of systemic lupus erythematosus. *Journal of Applied Genetics* **45**, 237–248.
71. Mansoor, F., Ali, A. and Ali, R. (2005) Binding of circulating SLE autoantibodies to oxygen free radical damaged chromatin. *Autoimmunity* **38**, 431–438.
72. Wuttge, D.M., Bruzelius, M. and Stemme, S. (1999) T-cell recognition of lipid peroxidation products breaks tolerance to self proteins. *Immunology* **98**, 273–279.
73. Toyoda, K., Nagae, R., Akagawa, M. *et al.* (2007) Protein-bound 4-hydroxy-2-nonenal: an endogenous triggering antigen of anti-DNA response. *Journal of Biological Chemistry* **282**, 25769–25778.
74. Lee, E., Furukubo, T., Miyabe, T. *et al.* (1996) Involvement of histone hyperacetylation in triggering DNA fragmentation of rat thymocytes undergoing apoptosis. *FEBS Letters* **395**, 183–187.
75. Lee, E., Nakatsuma, A., Hiraoka, R. *et al.* (1999) Involvement of histone phosphorylation in thymocyte apoptosis by protein phosphatase inhibitors. *IUBMB Life* **48**, 79–83.
76. Dieker, J.W., Fransen, J.H., van Bavel, C.C. *et al.* (2007) Apoptosis-induced acetylation of histones is pathogenic in systemic lupus erythematosus. *Arthritis and Rheumatism* **56**, 1921–1933.
77. Monks, T.J., Xie, R., Tikoo, K. *et al.* (2006) Ros-induced histone modifications and their role in cell survival and cell death. *Drug Metabolism Reviews* **38**, 755–767.
78. Elouaai, F., Lule, J., Benoist, H. *et al.* (1994) Autoimmunity to histones, ubiquitin, and ubiquitinated histone H2A in NZB × NZW and MRL-lpr/lpr mice. Anti-histone antibodies

are concentrated in glomerular eluates of lupus mice. *Nephrology Dialysis Transplantation* **9**, 362–366.
79. Ullrich, O., Sitte, N., Sommerburg, O. *et al.* (1999) Influence of DNA binding on the degradation of oxidized histones by the 20S proteasome. *Archives of Biochemistry and Biophysics* **362**, 211–216.
80. Ullrich, O., Reinheckel, T., Sitte, N. *et al.* (1999) Poly-ADP ribose polymerase activates nuclear proteasome to degrade oxidatively damaged histones. *Proceedings of the National Academy of Sciences USA* **96**, 6223–6228.
81. Kantengwa, S., Jornot, L., Devenoges, C. *et al.* (2003) Superoxide anions induce the maturation of human dendritic cells. *American Journal of Respiratory and Critical Care, Medicine* **167**, 431–437.
82. Decker, P., Kötter, I., Klein, R. *et al.* (2006) Monocyte-derived dendritic cells over-express CD86 in patients with systemic lupus erythematosus. *Rheumatology (Oxford)* **45**, 1087–1095.

Abstract

Anti-nucleosome autoantibodies (autoAbs) are detected in patients suffering from systemic lupus erythematosus (SLE), an inflammatory autoimmune disease. Nevertheless, the exact mechanisms leading to the production of those autoAbs are still not completely understood. Although genetic factors play a pivotal role in SLE development, additional triggers like environmental factors are also involved. Epigenetic factors have been suggested to contribute to lupus pathogenicity. Interestingly, oxidative stress participates in the generation of epigenetic alterations. This chapter reviews the contribution of oxidative stress and epigenetic factors in the context of anti-nucleosome autoAb induction during SLE and presents some mechanisms relevant to this particular clinical situation.

Key words: SLE; nucleosome; autoantibody; autoantigen; epigenetics; oxidative stress; immunogenicity; tolerance; pathogenicity; chromatin modification.

8

Stress, epigenetics and thyroid autoimmunity

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8.1 Introduction

Thyroid autoimmunity manifests clinically as chronic lymphocytic thyroiditis, known as Hashimoto thyroiditis and its variants (post-partum or sporadic thyroiditis), or as Graves' disease and atrophic thyroiditis. These autoimmune thyroid diseases (AITDs) occur as a result of aberrant immune response directed against the thyroid gland, when the immune system fails to recognize tolerance to self-antigens [1]. Evidence suggests that the phenotypic expression of AITD towards Graves' disease or Hashimoto thyroiditis is largely dependent on the pattern of immune response that predominates at a given time. This is likely determined by genetic factors or epigenetic effects, but may also be modified by environmental factors, among which stress appears to play an important role [2,3].

Stress exerts a profound effect on the immune system through neuroendocrine pathways. During a stress response, the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system are activated, resulting in systemic elevations in glucocorticoids and catecholamines, respectively. Both systems serve to counteract the effect of stressors and mainstain homeostasis [4].

It has long been thought that the stress hormones, and in particular the glucocorticoids, exert in general an immune-suppressive effect. However, accumulating evidence suggests that stress, through its effector neuroendocrine pathways, has a differential effect on the immune response, suppressing cellular and potentiating humoral immunity [5,6]. In this chapter, evidence is provided from experimental and clinical research in support of the hypothesis that stress, by affecting the balance of immune response, may influence the clinical expression of AITD.

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8.2 The Th1/Th2 balance in immune-response regulation

The type of immune response (cellular or humoral) is largely regulated by antigen-presenting cells (APCs) such as dendritic cells, macrophages and natural killer (NK) cells, which are components of innate immunity. Once a non-self-antigen is judged dangerous, the APC springs into action, presenting the (auto)antigen in conjunction with major histocompatibility complex (MHC) molecules and co-stimulatory signals to $CD4^+$ T helper (Th) cells. Depending on the signals they receive from APCs, naive $CD4^+$ Th cells are further differentiated into Th1 or Th 2 cells that are components of acquired immunity. In turn, Th1 cells secrete type 1 cytokines, including interferon-γ (IFN-γ), interleukin-2 (IL-2) and tumour necrosis factor α (TNF-α), which are pro-inflammatory and responsible for cell-mediated immunity. On the other hand, Th2 cells produce anti-inflammatory type 2 cytokines, primarily IL-4, IL-5, IL-10 and IL-13, and provide help to antigen-specific B lymphocytes to produce antibodies involved in humoral immunity [7,8].

Precursor $CD4^+$ Th cells are believed to be pluripotent and can follow a Th1 or Th2 differentiation pathway, depending on the cytokine environment they experience or the stimuli they encounter. Thus, if naive $CD4^+$ T cells are activated in the presence of IL-12 that is produced by APCs in response to antigenic stimulation, the transcription factor STAT4 will be activated, triggering the transcription of the gene for IFN-γ (*Ifng*). The production of IFN-γ leads to the transcriptional activation of target genes on T cells and the induction of key Th1-specific transcription factors such as T-bet. T-bet binds to regulatory elements at several loci, including *Ifng*, and transactivates *Ifng*, creating a positive-feedback loop. In contrast, IL-4, which is secreted mainly by $CD4^+$ T cells and mast cells, promotes the differentiation of Th2 cells and the production of type 2 cytokines, through activation of signal transducer and activator of transcription 6 (STAT) and the subsequent activation of the transcription factor GATA-3 [9,10] (Figure 8.1).

8.3 Stress hormones and the Th1/Th2 balance

The effect of glucocorticoids and catecholamines

Evidence suggests that glucocorticoids, at levels achieved during stress, suppress cell-mediated and potentiate humoral immunity. Glucocorticoids, acting through their cytoplasmic/nuclear receptors on APCs, suppress the production of IL-12, the main cytokine inducer of Th1 responses, and downregulate the expression of IL-12 receptors on T cells and natural killer cells, inhibiting IFN-γ production. On the other hand, glucocorticoids promote the production of IL-4 and IL-10 by Th2 cells. The latter could be the result of diminishing the restraining effect of IL-12 and IFN-γ on Th2 cells [11–14]. Additional evidence suggests that glucocorticoids may also block the transcriptional activity of T-bet, the key transcription factor in Th1-mediated cellular immunity [15].

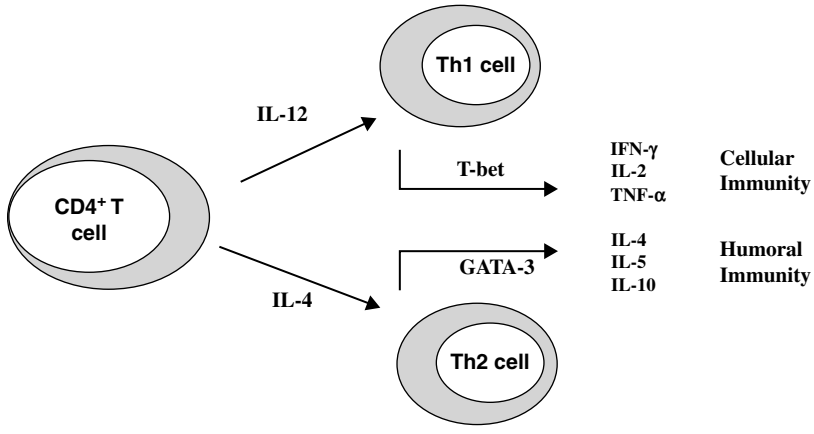

Figure 8.1 IL-12 and IL-4 direct the differentiation of naive $CD4^+$ T cells towards Th1 or Th2 subtypes, producing type 1 or type 2 cytokines respectively. Th2 cells express the transcription factor GATA-3 that induces expression of Th2-specific cytokines and inhibits IFN-γ production in developing Th1 cells. T-bet, a Th1-specific T-box transcription factor, induces IFN-γ and represses IL-4 and IL-5 production in Th2 cells.

In a similar way, catecholamines, acting on APCs through β2-adrenergic receptors, suppress the production of IL-12, thus inhibiting the differentiation of Th1 cells, while enhancing Th2 cell differentiation [16]. Furthermore, catecholamines appear to inhibit the production of TNF-α and potentiate the production of IL-10 by APCs [17,18]. Notably, β2-adrenergic receptors are expressed only on Th1 cells and not on Th2 cells, and this may provide an additional explanation for the differential effect of catecholamines on Th1/Th2 balance. Thus, β2-AR agonists inhibit IFN-γ production by Th1 cells but do not affect IL-4 production by Th2 cells [19]. Furthermore, glucocorticoids can enhance the sensitivity of peripheral mononuclear cells to the immunomodulatory effects of catecholamines, indicating cooperative effects of the stress hormones on the immune deviation of the type1/type2 cytokine balance in favour of type 2 expression [20]. In conclusion, both glucocorticoids and catecholamines, the main stress hormones, appear to cause selective suppression of cellular immunity and boost humoral immunity by downregulating type 1 and upregulating type 2 cytokine expression.

The effect of the local CRH-mast cell-histamine axis

In addition to the central hypothalamic corticotrophin-releasing hormone (CRH) that influences the type of immune response indirectly, through activation of the hypothalamic-pituitary-adrenal axis, CRH is also secreted locally at peripheral sites (peripheral or immune CRH) and may influence the immune system directly through local modulatory effects [21]. In this regard, the targets of immune CRH are the mast cells,

clusters of which are found in periarterial sympathetic plexuses and plexuses of nerve fibres within lymphoid parenchyma. Peripheral CRH activates mast cells, via a CRH type 1 receptor-dependent pathway, causing the release of histamine and other contents of mast cell granules. In turn, histamine acting via the H1 receptor may induce acute inflammation and allergic reactions, whereas acting through activation of H2 receptors may induce suppression of Th1 and a shift towards Th2 immune response [22,23]. Thus, the activation of the CRH-mast cell-histamine axis, through stimulation of H2 receptors, might influence the Th1/Th2 balance towards a Th2 phenotype.

The role of oxidative stress in Th1/Th2 balance

Glutathione in its reduced form (GSH) is the single most important regulatory antioxidant in cells. The intracellular ratio of GSH/oxidized glutathione (GSSG) is a useful measure of the cell's overall antioxidant status [24]. Studies in mice have shown that *in vivo* and *in vitro* depletion of GSH from APCs results in lower Th1 and higher Th2 activity, whereas GSH repletion may have the opposite effect [25]. In a similar way, macrophages with most of their glutathione in the reduced form become effectively type 1 cells capable of inducing Th1 differentiation, whereas macrophages with mostly GSSG are effectively type 2 cells and may induce Th2 responses [26]. It appears, therefore, that the immune response can exhibit a biased Th1 or Th2 phenotype depending on the relative antioxidant status of the APC, orchestrating this process.

Further studies have also demonstrated that high GSH inside the macrophage supports gene expression that leads to secretion of IL-12, the major Th1-polarizing cytokine. Furthermore, exposure of the macrophage to IFN-γ tends to raise its GSH, thereby reinforcing its orientation towards type 1 activity. Conversely, exposure of the macrophage to IL-4 lowers its GSH and steers it towards type 2 activity [27]. From these observations, it can be concluded that the antioxidant status at the level of APCs and their microenvironment can influence the ultimate pattern of immune response. Thus, conditions associated with increased oxidative stress at the cellular level may deplete APCs of antioxidants and direct their phenotypic orientation towards a Th2-type response.

8.4 The Th1/Th2 balance in thyroid autoimmunity

AITD, the most common-organ-specific autoimmune condition, may be clinically expressed as Hashimoto thyroiditis and its variants (post-partum or sporadic thyroiditis), Graves' disease and atrophic thyroiditis [28]. The main autoantigens in AITD are thyroglobulin, thyroid peroxidase (TPO) and the thyroid-stimulating hormone (TSH) receptor (TSHr). Hashimoto thyroiditis is characterized by lymphocytic infiltration of the thyroid parenchyma causing a diffuse or micronodular goitre in association

with positive anti-thyroid peroxidase and/or anti-thyroglobulin antibodies in serum and varying degrees of thyroid dysfunction. The intrathyroidal immune cells are T and B lymphocytes with the $CD4^+$ Th1 subtype predominating, although Th2 cells are also present [29]. Thyroid follicular cells in close proximity to the infiltrating lymphocytes appear apoptotic, suggesting immune-mediated apoptotic destruction of these cells [30]. On the other hand, in Graves' disease there is follicular cell hypertrophy and hyperplasia along with hyperfunction, manifesting as a diffuse goitre and hyperthyroidism. The lymphocytic infiltration of the thyroid is patchy and not as massive as in Hashimoto thyroiditis and the majority of T lymphocytes are $CD4^+$ Th2 cells, although Th1 cells are also found. TSHr-stimulating antibodies are the hallmark, being responsible for thyroid follicular growth and hyperfunction [29,31]. On the other hand, atrophic thyroiditis is rare and characterized by a small (atrophic) thyroid gland and clinical hypothyroidism. TSHr-blocking antibodies are present in up to 50% of patients with atrophic thyroiditis and these may be responsible for thyroid-gland atrophy and hypofunction [32].

Studies in animal models suggest that thyroid autoimmunity is a three-stage process [33]. In the first stage, there is an increased appearance of intrathyroidal APCs, which take up and present thyroid autoantigens, together with MHC class II antigens and co-stimulatory molecules, to $CD4^+$ T cells. The second stage involves the interaction of the Th lymphocytes with the presented autoantigen. If immune tolerance and its regulation by co-stimulatory signals are lost, then inappropriate activation of antigen-specific T lymphocytes takes place, leading to differentiation and clonal expansion of autoreactive $CD4^+$ Th cells and antibody-producing B lymphocytes. In the final stage, the generated autoreactive T and B lymphocytes accumulate in large numbers in the thyroid parenchyma. The latter then becomes 'a battlefield' with infiltrating lymphocytes and defending thyrocytes fighting for survival. The outcome of this interaction determines the different clinical phenotype of AITD. This is largely dependent on the Th1/Th2 balance and the pattern of cytokines released in the local microenvironment [1,2].

Predominance of a Th1-type immune response, favouring cell-mediated immunity, may create a pro-apoptotic milieu for the thyroid cells. Fas and/or TNF-related apoptosis-inducing ligand (TRAIL)-dependent apoptotic pathways are activated by type-1 pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-2, and the thyroid cells undergo apoptosis, leading to Hashimoto thyroiditis or its variants [34,35]. A predominant Th2 activity, which favours humoral immunity, may induce antigen-specific B lymphocytes to produce anti-TSHr antibodies and create an anti-apoptotic potential for the thyroid follicular cells. If the prevailing type of anti-TSHr antibodies is stimulatory, thyroid cell hyperplasia and hyperfunction ensue, leading to Graves' hyperthyroidism. If, on the other hand, TSHr-blocking antibodies predominate, then thyroid cell atrophy and hypofunction occur, leading to atrophic thyroiditis (Figure 8.2). Thus, the phenotypic expression of AITD towards Graves' disease or Hashimoto thyroiditis is dependent on the balance of Th1 and Th2 immune responses regulated by APCs and the type of cytokines predominating in the thyroid parenchyma. The fact that both conditions may develop in the same individual at different times

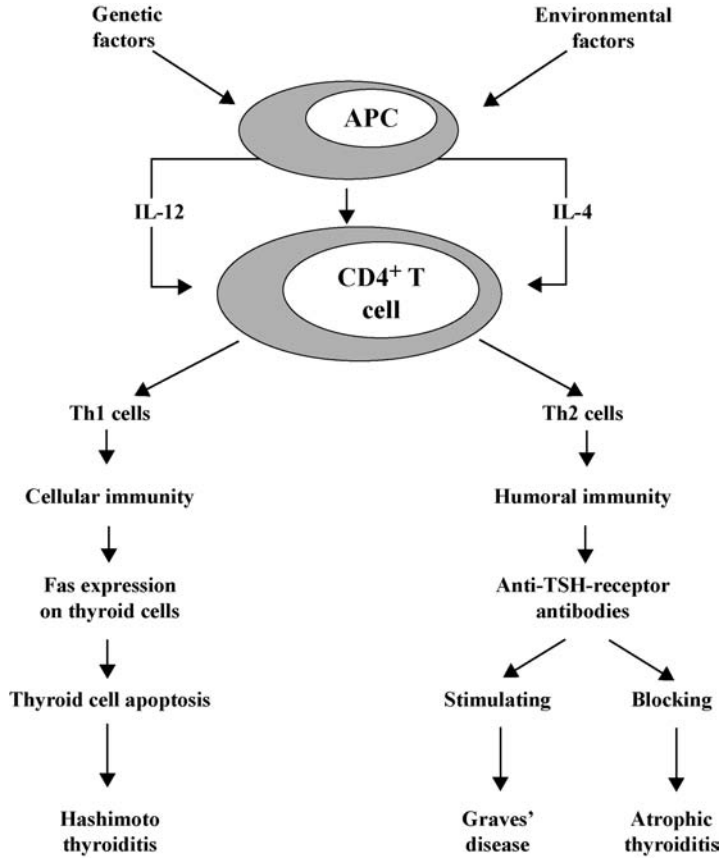

Figure 8.2 Hypothetic role of the Th1/Th2 balance in the clinical expression of autoimmune thyroid diseases. Predominance of Th1 lymphocytes producing pro-inflammatory cytokines may induce thyroid cell apoptosis leading to Hashimoto thyroiditis. Conversely, predominance of Th2 cells, favouring humoral immunity, may lead to production of stimulating or blocking anti-TSHr antibodies and result in the clinical expression of Graves' disease or atrophic thyroiditis, respectively.

suggests that the Th1/Th2 balance is a dynamic process that may be influenced by exogenous factors acting on APCs.

8.5 Association of stress with thyroid autoimmunity

The first clinical observation of an association between stressful life events and hyperthyroidism was made by Parry in 1825 along with the description of the disease, and subsequently by Graves, von Basedow and others [36]. Both physical stress, such as trauma or major illness, and psychological stress, such as bereavement, have been implicated. These early reports were followed by epidemiological observations

of an increased incidence of hyperthyroidism during major wars, a condition named *kriegsbasedow* [37]. Thus, the incidence of hyperthyroidism in the Danish population increased significantly during the 1941–1945 German occupation. Also, hospital admissions for thyrotoxicosis in occupied Scandinavian countries increased 5–6-fold during the 1939–1945 war, and returned to normal rates afterwards [37]. More recent evidence for such an association was the 5-fold increase in Graves' disease compared with toxic nodular goitre that was observed during the civil war in former Yugoslavia [38]. Other studies failed to show an increase in anti-thyroid drug use during the civil unrest in Northern Ireland [39] or an increase in stressful life events in consecutive thyrotoxic patients attending an outpatient's clinic [40]. However, the latter reports can be criticized for failing to distinguish between Graves' disease and other causes of thyrotoxicosis. In an earlier study, Forteza found that most of his 115 patients experienced stressful events just before the first signs of Graves' disease [41]. Following these early clinical observations a number of formal case-control studies and population-based surveys using self-rated questionnaires have examined the effect of stress on the onset or the clinical course of Graves' thyrotoxicosis.

Stress and the onset of Graves' disease

The first large population-based clinical study that established an association between stressful life events and the onset of Graves' disease was from Sweden. Using a self-rated questionnaire, 208 patients with newly diagnosed Graves' disease were found to have more negative life events and higher negative life-event scores in the year preceding the diagnosis, compared to 372 controls [42]. Subsequent case-control studies in different ethnic populations confirmed the association of stressful life events with the onset of Graves' disease [43,45]. One study from Japan reported an association of stress with Graves' disease in women but not in men [46]. The above studies have been criticized because of their retrospective nature, the influence of recall bias, and the fact that thyrotoxicosis itself might also manifest with anxiety symptoms and negative emotional events, raising the question of distinguishing between cause and effect [47,48]. This problem was partially addressed by a more recent study that compared Graves' disease patients with patients having toxic nodular goitre and healthy controls, in order to correct for the effect of thyrotoxicosis. A significant increase in the number of negative life events was found in the Graves' disease patients compared to those with toxic nodular goitre and normal controls [49]. This finding supports the notion that stress may precipitate autoimmune as opposed to non-autoimmune hyperthyroidism.

Stress and the clinical course of Graves' disease

Few studies have examined the effect of stress on the clinical course of Graves' disease. In a retrospective study, treatment with a benzodiazepine, in addition to drug therapy, reduced the relapse rate of thyrotoxicosis from 74% in untreated patients to 29%

in patients treated with benzodiazepine, suggesting that stress management was effective in improving the prognosis of Graves' disease [50]. Two prospective case-control studies also suggest that stress may have a negative impact on the outcome of Graves' disease. The first study from Japan investigated the outcome of patients with newly diagnosed Graves' disease after 12 months of anti-thyroid drug therapy in relation to stressful life events. The authors reported that 'daily stresses' at 6 months after starting therapy were associated with continued hyperthyroid state 12 months later [51]. This effect was seen only in women, however, as the number of males in the study was too small to reach a significant effect. In a more recent study, it was shown that the relapse rate after anti-thyroid drug treatment for Graves' disease was related to daily stresses and some personality traits, and that stress scores correlated with the level of anti-TSHr antibodies after cessation of anti-thyroid drugs [52]. The findings from these studies, although limited in number, indicate that stress may also affect the clinical course of Graves' disease.

Apart from the epidemiological observations and clinical studies stated above, there are also several case reports that support a relationship between stress and the onset or outcome of Graves' disease. Misaki *et al.* reported three cases of Graves' hyperthyroidism occurring after partial thyroidectomy for papillary carcinoma [53]. The authors suggested that surgical stress might alter immune homeostasis, converting preclinical into clinical Graves' hyperthyroidism. A relationship between stress and the onset and clinical course of Graves' disease has also been reported in children. Morillo and Gardner reported four children in whom a 'separation' event was related to the onset or relapse of Graves' disease [54]. We have reported five patients who developed mild autoimmune hyperthyroidism following a major stressful life event such as bereavement, job loss, stress at work and major surgery [55]. In all cases, the hyperthyroidism followed a short course (up to 6 months) on small doses of anti-thyroid drugs and went into remission as the stress situation resolved.

Stress and Hashimoto thyroiditis

In contrast to Graves' disease, few studies have examined the association between stress and Hashimoto thyroiditis. Two case-control studies evaluated the role of stressful events in Hashimoto thyroiditis or post-partum thyroiditis. They concluded that stress was not a trigger in either condition [56,57]. A recent population study also did not find a relationship between stressful life events and the presence of anti-thyroid peroxidase antibodies among euthyroid women [58]. The onset and clinical course of Hashimoto thyroiditis are often insidious and the diagnosis may be delayed until the patients develop overt hypothyroidism, making it difficult to assess the role of stress in the onset and natural history of the disease. In summary, the weight of evidence from the available epidemiological observations and clinical studies suggest an association between stress and Graves' disease but not with Hashimoto thyroiditis. The biological mechanisms underlying the association of stress with Graves' disease are not known, but a possible explanation is given below.

8.6 Stress in the clinical expression of thyroid autoimmunity: a unifying hypothesis

Evidence from animal studies and clinical observations suggests that a hyperactive or hypoactive stress response may be associated with decreased or increased vulnerability to different types of autoimmune disease [59]. Thus, Fisher rats, which have a hyperactive stress system, are resistant to experimentally induced Th1-mediated autoimmune diseases, such as rheumatoid arthritis, uveitis and experimental allergic encephalomyelitis. Conversely, Lewis rats, which have a hypoactive hypothalamic-pituitary-adrenal axis, are prone to develop Th1-mediated autoimmune conditions [59].

A natural condition with changes in the Th1/Th2 balance is pregnancy and the post-partum period. Pregnancy results in suppression of Th1-mediated cellular immune activity and preservation or enhancement of Th2-mediated humoral immunity. In the third trimester, Th1-type cytokines such as IFN-γ and IL-2 decline and Th2 cytokines, in particular IL-4, increase. This shift may permit the histoincompatible fetal/placental unit to avoid rejection by a cell-mediated immune attack from the mother [60,61]. These immune changes in pregnancy develop in parallel with a marked increase in glucocorticoids along with increases in oestrogen and progesterone. Progesterone appears to cause a shift in Th1/Th2 balance similar to that described for glucocorticoids [62]. Indeed, the changes in the hormonal milieu may explain why pregnant women experience remission of Th1-mediated autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, type 1 diabetes and autoimmune thyroiditis, and aggravation of Th2-mediated autoimmune conditions such as systemic lupus erythematosus [63–65].

In the post-partum period, the hormonal milieu changes abruptly, with glucocorticoids, oestrogens and progesterone decreasing to subnormal levels, allowing a prompt recovery of cell-mediated immune function [64]. This Th2-to-Th1 'return shift' might explain the increase in the incidence of post-partum thyroiditis and other Th1-mediated autoimmune conditions. Analogous clinical situations associated with a decreased stress system activity are seen during the period that follows cure from Cushing's syndrome or discontinuation of glucocorticoid therapy [66,67]. These situations have been associated with increased susceptibility to Th1-mediated immune disorders. A similar rebound reaction may occur in periods that follow cessation of chronic stress. On the other hand, Graves' disease and other Th2-predominal conditions are frequently associated with allergic diseases [68]. Further support for the importance of the Th2 pathway in Graves' disease comes from observations that recurrence after anti-thyroid drug therapy is more likely following an attack of allergic rhinitis and elevated IgE levels, which is a marker of Th2 activity [69]. Interestingly, in this regard, humanized anti-CD52 monoclonal antibody therapy for multiple sclerosis, which causes change in the immune response from Th1 to Th2 phenotype, was reported to trigger the development of Graves' disease [70].

On the basis of these observations and the information cited above, a unifying hypothesis for the role of stress in the clinical expression of AITD can be formulated (Figure 8.3). Environmental factors interacting with genetic factors may induce an aberrant immune response against thyroid autoantigens and render an individual susceptible to develop thyroid autoimmunity. The potential for precursor $CD4^+$ T lymphocytes to differentiate towards Th1 or Th2 type activity following interaction with APCs is an important branch point for the development of cell-mediated or humoral autoimmunity. If such an individual is under severe stress, the stress hormones may steer the balance towards a Th2-type phenotype. Effector Th2 cells and type 2 cytokines will then induce antigen-specific B lymphocytes to produce anti-TSHr antibodies. The concomitant suppression of the Th1-effector pathway will protect thyroid cells from a cell-mediated immune attack. Under these circumstances, the clinical outcome will be Graves' disease. Conversely, if a susceptible person is recovering from a stress response or the immune-suppressive effect of pregnancy, a rebound reaction may reactivate the Th1-mediated branch pathway, leading to

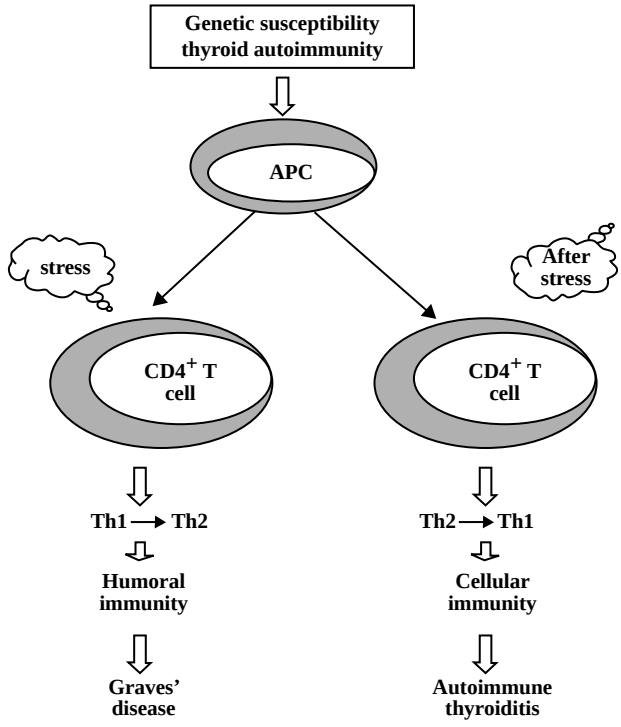

Figure 8.3 Hypothetic role of stress in the clinical expression of autoimmune thyroid diseases. In individuals susceptible to thyroid autoimmunity, severe stress may influence the Th1/Th2 balance towards a Th2-type response and humoral immunity, resulting in the expression of Graves' disease. After stress, a Th2-to-Th1 shift induces cell-mediated immunity against thyroid cells, leading to the development of Hashimoto thyroiditis.

cellular immune response and apoptotic destruction of thyroid follicular cells by the pro-inflammatory cytokines. The likely outcome will then be autoimmune (sporadic) or post-partum thyroiditis, respectively. Epigenetic mechanisms may be involved in this process, as discussed in the following section.

8.7 Epigenetic regulation of T cell differentiation and stress hormones

Epigenetic regulation of gene expression is an important mechanism that controls transcriptional activation or repression of specific gene loci. Methylation of CpGs is generally associated with transcriptional inactivity within a gene locus, whereas removal of the methyl group from cytosine signals a shift from inert chromatin to active or open loci [71]. A growing body of research has examined and implicated DNA methylation as a significant regulator of lineage commitment and specific cytokine production during Th cell differentiation [72]. Studies of mice deficient in the methyltransferase Dnmt 1 indicate an important and selective contribution of DNA methylation to cell-lineage-specific gene expression in developing and mature T cells [73].

The differentiation of naive $CD4^+$ T lymphocytes along the Th1 or Th2 differentiation pathway is thought to be regulated by epigenetic mechanisms leading to activation or silencing of the loci encoding type-2 cytokines (IL-4, IL-13) or the locus encoding IFN-γ, respectively [74]. A general model proposes that naive T cells have the potential to differentiate into either Th1 or Th2 cells. Following induction of the differentiation programme, the transcriptional rates of IL-4 and IFN-γ genes diverge. The Th1 differentiation programme leads to an increase in the transcriptional activity of the IFN-γ gene and the silencing of the genes for IL-4, IL-5 and IL-13. The converse is true for the Th2 differentiation programme, which leads to increased transcriptional activity of IL-4, IL-5 and IL-13 genes, and silencing of the IFN-γ gene. Key stimulators of the Th1 differentiation programme include IL-12 and the transcription factor STA4 and the IFN-γ/STAT1-trascription factor T-bet signalling pathways, whereas stimulation of the Th2 differentiation programme includes the IL-4/STAT6 and the transcription factor GATA-3 signalling pathway [75].

It therefore appears that specific epigenetic changes regulate the activation of the IFN-γ gene during the process of differentiation of $CD4^+$ T cells into Th1 cells and also the silencing of the same gene in differentiating Th2 cells [74]. Recent evidence suggests that specific histone-methylation patterns of chromatin surrounding the IFN-γ gene could explain the permissive chromatin environment for IFN-γ gene expression in differentiated Th1 cells relative to the non-permissive chromatin environment for IFN-γ gene expression in Th2 cells [76]. The dynamic nature of the epigenetic changes that drive the silencing of IFN-γ gene is dependent on two transcriptional activators of Th2 cell differentiation: GATA-3 and STAT6. In Th1 cells the establishment of histone-acetylation marks across the IFN-γ gene locus is dependent on STAT4 and T-bet [77]. Glucocorticoid hormones bind to glucocorticoid receptors, which act as transcription

regulators of many genes. Among these genes of particular interest is DNA (cytokine-5)-methytransferase 1, which is involved in preservation of DNA methylation patterns as well as in *de novo* DNA methylation, and is important in epigenetic transcription repression [78]. Recent reports suggest that glucocorticoids can modulate Dnmt 1 expression [79]. There is also experimental evidence suggesting that the activated glucocorticoid receptor inhibits T-bet by direct protein–protein interaction and that glucocorticoid treatment reduces T-bet and STAT1 expression in mononclear cells [15,80]. It is likely, therefore, that stress hormones and in particular glucocorticoids may influence the phenotypic expression of thyroid autoimmunity towards Graves' disease through epigenetic modification of T cell differentiation. However, the exact mechanism involved in this process awaits further elucidation.

8.8 Conclusions

Circumstantial evidence suggests that Hashimoto thyroiditis and Graves' disease, the two opposite clinical entities of AITD, manifest different immune phenotypes. Hashimoto thyroiditis is predominately a Th1-mediated autoimmune disease, whereas Graves' disease has a predominant Th2 phenotype. There is convincing evidence from epidemiological and clinical studies supporting the view that stress may favour the development of Graves' disease, but there is limited information on the role of stress in Hashimoto thyroiditis. However, whether stress plays a causative role in the development of Graves' disease is not yet clear. It is likely that, in susceptible individuals, stress hormones may influence the clinical expression of thyroid autoimmunity towards the development of Graves' disease by suppressing cellular immunity and potentiating humoral immunity. Epigenetic mechanisms may be involved in this process. On the other hand, recovery from stress, through a rebound effect of cellular immunity, may favour the development of autoimmune thyroiditis. This, however, is only a working hypothesis based on circumstantial evidence and needs further substantiation. Future studies also need to focus on identifying agents that can restore the type 1/type 2 cytocine balance, in the presence of stress hormones, and serve as a basis for interventions to prevent the stress-associated immune conditions in humans.

References

1. Weetman, A.P. (2004) Cellular immune responses in autoimmune thyroid disease. *Clinical Endocrinology* **61**, 405–413.
2. Fountoulakis, S. and Tsatsoulis, A. (2004) On the pathogenesis of autoimmune thyroid disease: a unifying hypothesis. *Clinical Endocrinology* **60**, 397–409.
3. Prummel, M.F., Strieder, T. and Wiersinga, W.M. (2004) The environment and autoimmune thyroid diseases. *European Journal of Endocrinology* **150**, 605–618.
4. Chroussos, G.P. (1998) Stressors, stress and neuroendocrine integration of the adaptive response. The 1997 Hans Selye memorial lecture. *Annals of New York Academy of Sciences* **851**, 311–315.

5. Elenkor, I.J. and Chroussos, G.P. (1999) Stress hormones, Th1-Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease. *Trends in Endocrinology and Metabolism* **13**, 567–581.
6. Rook, G.A.W. (1999) Glucocorticoids and immune function. *Bailliere's Clinical Endocrinology and Metabolism* **13**, 567–581.
7. Abbas, A.K., Murphy, W.K.M. and Sher, A. (1996) Functional diversity of helper T-lymphocytes. *Nature* **383**, 787–795.
8. Fearon, D.T. and Locksley, R.M. (1996) The instructive role of innate immunity in the acquired immune response. *Science* **272**, 50–53.
9. Murphy, K.M. and Reiner, S.L. (2002) The lineage decisions of helper T cells. *Nature Reviews Immunology* **2**, 935–944.
10. Szabo, S.J., Sullivan, B.M., Peng, S.L. *et al.* (2003) Molecular mechanisms regulating Th1 immune responses. *Annual Reviews in Immunology* **21**, 713–758.
11. Blotta, M.H., Rekruyff, R.H. and Umetsu, D.J. (1997) Gorticosteroids inhibit IL-12 production in human monocytes and enhance their capacity to induce IL-4 synthesis in CD4 + lymphocytes. *Journal of Immunology* **158**, 5589–5595.
12. Wu, C.Y., Wang, J.F., Dyer, M.C. *et al.* (1998) Prostaglandin E2 and dexamethasone inhibit IL-12 receptor expression and IL-12 responsiveness. *Journal of Immunology* **161**, 2733–2730.
13. Ramivez, F., Fowell, B.J., Puklavec, M. *et al.* (1990) Glucocorticoids promote a Th2 cytokine response by CD4 + T cells in vitro. *Journal of Immunology* **156**, 2406–2412.
14. Agarwal, S.K. and Marshall, G.D. (2001) Dexamethasone promotes type 2 cytokine production primarily through inhibition of type 1 cytokines. *Journal of Interferon and Cytokine Research* **21**, 147–155.
15. Liberman, A.C., Refojo, O., Druker, J. *et al.* (2007) The activated glucocorticoid receptor inhibits the transcription factor T-bet by direct protein interaction. *FASEB Journal* **21**, 1177–1188.
16. Elenkov, I.J., Papanikolaou, D.A., Wilder, R.L. *et al.* (1996) Modulatory effects of glucocorticoids and catecholamines in human interleukin-12 and interleukin-10 production: clinical implications. *Proceedings of the Association of American Physicians* **108**, 374–381.
17. Panina-Bordignon, P., Mazzed, D., Lucia, P.D. *et al.* (1997) Beta 2-agonists prevent Th1 development by selective inhibition of interleukin-12. *Journal of Clinical Investigation* **100**, 1513–1519.
18. Agarwal, S.K. and Marshall, G.D. (2000) β-Adrenergic modulation of human type 1/type 2 cytokine balance. *Journal of Allergy and Clinical Immunology* **105**, 91–98.
19. Sanders, V.M., Baker, R.A., Ramer-Quinn, D.S. *et al.* (1997) Differential expression of beta 2-adrenergic receptor by Th1 and Th2 clones: implications for cytokine production and B-cell help. *Journal of Immunology* **158**, 4200–4210.
20. Salicru, A.N., Sams, C.F. and Marshall, G.D. (2007) Coooperative effects of corticosteroids and catecholamines upon immune deviation of the type-1/type-2 cytokine balance in favor of type-2 expression in human peripheral blood mononuclear cells. *Brain, Behavior and Immunity* **21**, 913–920.
21. Karalis, K., Savo, H., Redwine, I. *et al.* (1991) Autocrine and paracrine inflammatory actions of corticotrophin-releasing hormone in vivo. *Science* **254**, 421–423.
22. Webster, E.L., Torpy, D.J., Elenkov, I.J. and Chroussos, G.P. (1998) Corticotropin-releasing hormone and inflammation. *Annals of New York Academy of Sciences* **840**, 21–23.
23. Elenkov, I.J., Webster, E.L., Papanikolaou, D.A. *et al.* (1998) Histamine potently suppresses human IL-12 and stimulates IL-10 production via H2 receptors. *Journal of Immunology* **161**, 2586–2593.
24. Kidd, P.M. (1997) Glutathione: systemic protectant against oxidative and free radical damage. *Alternative Medicine Reviews* **2**, 155–176.
25. Peterson, J.D., Herzenberg, L.A., Vasquez, K. and Waltenbaugh, C. (1998) Glutathione levels in antigen-presenting cells modulateTh1 versus Th2 response patterns. *Proceedings of National Academy of Sciences USA* **95**, 3071–3076.

26. Murata, Y., Shimamura, T. and Hamuro, J. (2002) The polarization of Th1/Th2 balance is dependent on the intracellular thior redox status of macrophages due to distinctive cytokine production. *International Immunology* **14**, 201–212.
27. Murata, Y., Shimamura, T., Tagami, T. *et al.* (2002) The skewing to Th1 induced by lentinan is directed through the distinctive cytokine production by macrophages with elevated intracellular glutathione content. *International Immunopharmacology* **2**, 673–689.
28. Pearce, E.N., Farwell, A.P. and Braverman, L.E. (2003) Current concepts: thyroiditis. *New England Journal of Medicine* **348**, 2640–2655.
29. Roura-Mir, C., Catalf, M., Sospedra, M. *et al.* (1997) Single-cell analysis of intrathyroidal lymphocytes shows differential cytokine expression in Hashimoto's and Graves' disease. *European Journal of Endocrinology* **271**, 3290–3302.
30. Kotani, T., Aratake, Y., Hiral, K. *et al.* (1995) Apoptosis in thyroid tissue from patients with Hashimoto's thyroiditis. *Autoimmunity* **20**, 231–236.
31. Heuer, M., Aust, G., Ode-Haim, S. and Scherbaum, W.A. (1990) Different cytokine mRNA profile in Graves' disease, Hashimoto's thyroiditis and in immune thyroid disorders determined, by quantitative reverse transcriptase polymerase chain reaction (RT-PCR). *Thyroid* **6**, 97–105.
32. Orgiazzi, J. (2000) Anti-TSH receptor antibodies in clinical practice. *Endocrinology and Metabolism Clinics of North America* **29**, 339–355.
33. Ruwhof, C. and Drexhage, H.A. (2001) Iodine and thyroid autoimmune disease in animal models. *Thyroid* **11**, 427–436.
34. Bretz, J.D. and Baker, J.R. (2001) Apoptosis and autoimmune thyroid disease: following a TRAIL to thyroid destruction? *Clinical Endocrinology* **55**, 1–11.
35. Andrikoula, M. and Tsatsoulis, A. (2001) The role of Fas-mediated apoptosis in thyroid disease. *European Journal of Endocrinology* **144**, 561–568.
36. Rosch, P.J. (1993) Stressful life events and Graves' disease. *Lancet* **341**, 566–567.
37. Gorman, C.A. (1990) Environment and Autoimmunity: a critical review of the role of stress in hyperthtroidism. In: Drexhage H.A., de Vijlder J.M.M., Wiersinga W.M. (eds), *The Thyroid Gland*, pp. 191–200. Elsevier Science Publishers, Amsterdam.
38. Paunkovic, N., Paunkovic, J., Paulovic, O. and Paunkovic, Z. (1998) The significant increase in incidence of Graves' disease in eastern Servia during the civil war in the former Yugoslavia (1999 to 1995). *Thyroid* **8**, 37–41.
39. Hadden, D.R. and McDevitt, D.G. (1974) Environmental stress and thyrotoxicosis: absense of association. *Lancet* ii, 577–578.
40. Gray, J. and Hofenberg, R. (1985) Thyrotoxicosis and stress. *Quarterly Journal of Medicine* **54**, 153–160.
41. Forteza, M.E. (1973) Precipitating factors in hyperthyroidism. *Geriatrics* **28**, 123–126.
42. Winsa, B., Adami, H., Berqstrom, R. *et al.* (1991) Stressful life events and Graves' disease. *Lancet* **338**, 1475–1478.
43. Sovino, N., Girelli, M.E., Boscato, M. *et al.* (1993) Life events in the pathogenesis of Graves' disease. A controlled study. *Acta Endocrinologica* **128**, 293–296.
44. Kung, A.W. (1995) Life events, daily stresses and coping in patients with Graves' disease. *Clinical Endocrinology* **42**, 303–308.
45. Rudosavljevic, V.R., Jankovic, S.M. and Marinkovic, J.M. (1996) Stressful life events in the pathogenesis of Graves' disease. *European Journal of Endocrinology* **134**, 699–701.
46. Yoshiuchi, K., Kumano, H., Nomura, S. *et al.* (1998) Stressful life events and smoking were associated with Grave's disease in women, but not in men. *Psychosomatic Medicine* **60**, 182–185.
47. Chiovato, L. and Pinchera, A. (1996) Stressful life events and Graves' disease. *European Journal of Endocrinology* **134**, 680–682.
48. Dayan, C.M. (2001) Stressful life events and Graves' disease revisited. *Clinical Endocrinology* **55**, 13–14.
49. Matos-Santos, A., Nobile, E.L. and Costa, J.G.E. (2001) Relationship between the number and impact of stressful life events and the onset of Graves' disease and toxic nodular goitre. *Clinical Endocrinology* **55**, 15–19.
50. Benvenga, S. (1996) Benzodiazepines and remission of Graves' disease. *Thyroid* **6**, 659–600.

51. Yoshiuchi, K., Kumano, H., Nomura, S. *et al.* (1998) Psychosocial factors influence the short-term outcome of antithyroid drug therapy in Graves' disease. *Psychosomatic Medicine* **60**, 592–596.
52. Fukao, A., Takamatsu, J., Marakami, J.Y. *et al.* (2001) The relationship of psychological factors to the prognosis of hyperthyroidism in antithyroid drug-treated patients with Grave's disease. *Clinical Endocrinology* **58**, 550–555.
53. Misaki, I., Iwata, M., Kasagi, K. *et al.* (2000) Hyperthyroid Graves' disease after hemithyroidectomy for papillary carcinoma: report of three cases. *Endocrine Journal* **47**, 191–195.
54. Morillo, E. and Gardner, L.I. (1979) Bereavement as an antecedent factor in thyrotoxicosis of childhood: four case studies with survey of possible metabolic pathways. *Psychosomatic Medicine* **41**, 545–555.
55. Tsatsoulis, A. and Panteli, K. (1996) Stress-induced mild thyrotoxicosis. *European Journal of Internal Medicine* **7**, 247–250.
56. Martin-du Pan, R.C. (1998) Triggening role of stress and pregnancy in the accurence of 98 cases of, Grave's disease compared to 95 cases of Hashimoto's thyroiditis and 97 cases of thyroid nodules. *Annals of Endocrinology (Paris)* **59**, 107–112.
57. Oretti, R.C., Harris, B. and Lazaros, J.H. (2002) Is there an association between life events, postnatal depression and thyroid dysfunction in thyroid antibody positive women? *International Journal of Social Psychiatry* **49**, 70–76.
58. Streder, I.G.A., Prummel, M.F., Tussen, J.G.P. *et al.* (2005) Stress is not associated with thyroid peroxidase autoantibodies in euthyroid women. *Brain Behaviour and Immunity* **19**, 2003–2006.
59. Wilder, R.L. (1995) Neuroendocrine-immune system interaction and autoimmunity. *Annual Reviews of Immunology* **83**, 2003–2006.
60. Elenkov, I.J., Hoffman, J. and Wilder, R.C. (1997) Does differential neuroendocrine control of cytokine production govern the expression of autoimmune diseases in pregnancy and the postpartum period? *Molecular Medicine Today* **3**, 379–383.
61. Raghupathy, R. (1997) Th1-type immunity is incompatible with succesful pregnancy. *Immunology Today* **18**, 478–482.
62. Miyaura, H. and Iwata, M. (2002) Direct and indirect inhibition of Th1 development by progesterone and glucocorticoids. *Journal of Immunology* **168**, 1087–1094.
63. Muller, A.F., Drexhage, H.A. and Berghout, A. (2001) Postpartum thyroiditis and autoimmune thyroiditis in women of childbearing age: recent insights and consequences for artenatal and postnatal care. *Endocrine Reviews* **22**, 605–630.
64. Davies, T.F. (1999) The thyroid immunology of the postpartum period. *Thyroid* **9**, 675–684.
65. Hall, R. (1995) Pregnancy and autoimmune endocrine disease. *Ballieres Clinical Endocrinology and Metabolism* **9**, 137–155.
66. Takasu, N., Komiya, I., Nugasawa, Y. *et al.* (1990) Exacerbetion of autoimmune thyroid dysfunction after unilateral adrenalectomy in patients with Cushing's Syndrome due to an adrenocortical adenoma. *New England Journal of Medicine* **322**, 1708–1712.
67. Takasu, N., Ohara, N., Yamada, T. and Komiya, I. (1993) Development of autoimmune thyroid dysfunction after bilateral adrenalectomy in a patient with Carney's complex and after removal of, A.C.TH producing adenoma in a patient with Cushing's disease. *Journal Endocrinological Investigation* **16**, 691–702.
68. Amimo, N., Hidaka, Y., Takano, T. *et al.* (2003) Association of seasonal allergic rhinitis is high in Graves' disease and low in painless thyroiditis. *Thyroid* **13**, 811–814.
69. Sato, A., Takemura, Y., Tamada, T. *et al.* (1999) A possible role of immunoglobulin E in patients with hyperthyroid Graves' disease. *Journal of Clinical Endocrinology and Metabolism* **84**, 3602–3605.
70. Coles, A.J., Wing, M., Smith, S. *et al.* (1999) Pulsed monoclonal antibody treatment and autoimmune thyroid disease in multiple sclerosis. *Lancet* **354**, 1961–1965.
71. Ng, H.-N. and Bird, A. (1999) DNA methylation and chromatin modification. *Current Opinion in Genetics and Development* **9**, 158–163.
72. Lee, G.R., Kim, S.T., Spilianakis, C.G. *et al.* (2006) T helper cell differentiation: regulation by cis elements and epigenetics. *Immunity* **24**, 369–379.

73. Lee, P.P., Fitzpatrick, D.R., Beard, L. *et al.* (2001) A critical role of Dnmt 1 and, D.N.A methylation in T cell development, function, and survival. *Immunity* **15**, 763–774.
74. Spilianakis, C.G. and Flavell, R.A. (2007) Epigenetic regulation of *Ifng* expression. *Nature Immunology* **8**, 681–683.
75. Ansel, K.M., Lee, D.U. and Rao, A. (2003) An epigenetic view of helper T cell differentiation. *Nature Immunology* **4**, 616–623.
76. Schoenborn, J.R., Dorschner, M.O., Sekimata, M. *et al.* (2007) Comprehensive epigenetic profiling identifies multiple distal regulatory elements directing transcription of the gene encoding interferon-γ. *Nature Immunology* **8**, 732–742.
77. Chang, S. and Aune, T.M. (2007) Dynamic changes in histone-methylation 'marks' across the locus encoding interferon-γ during the differentiation of T helper type 2 cells. *Nature Immunology* **8**, 723–731.
78. Goyal, R., Reinhhardt, R. and Teltsch, A. (2006) Accuracy of DNA methylation pattern preservation by the Dnmt 1 methyltransferase. *Nucleic Acids Research* **34**, 1182–1188.
79. Ichinose, M., Miki, K., Tatematsu, M. *et al.* (1990) Hydrocortisone induced enhancement of expression and changes in methylation of pepsinogen genes in stomach mucosa of the developing rat. *Biochemical and Biophysical Research Communications* **172**, 1086–1093.
80. Frisullo, G., Nociti, V., Lorio, R. *et al.* (2007) Glucocorticoid treatment reduces T-bet and pSTAT1 expression in monuclear cells from relapsing remitting multiple sclerosis patients. *Clinical Immunology* **124**, 284–293.

Abstract

Stress hormones may influence the differentiation of naive T helper (Th) cells away from Th1 and towards a Th2 phenotype, resulting in suppression of cell-mediated immunity and potentiation of humoral immune response. Thyroid autoimmunity manifests clinically as Hashimoto thyroiditis or as Graves' disease. This differential phenotypic expression is largely dependent on the balance of Th1 and Th2 immune responses. Predominance of Th1 cells producing pro-inflammatory cytokines may cause destruction of thyroid cells, leading to Hashimoto thyroiditis. Conversely, predominance of Th2 cells and type 2 cytokines may induce the production of thyroid-stimulating hormone receptor-stimulating antibodies, causing Graves' disease. Epidemiological studies support an association between stress and Graves' disease. In susceptible individuals, stress may favour the development of Graves' disease by shifting the Th1/Th2 balance towards Th2. After recovery from stress or the immune-suppressive effect of pregnancy, a Th2-to-Th1 'return shift' may induce the development of autoimmune or post-partum thyroiditis, respectively. Epigenetic mechanisms are likely to be involved in this process.

Key words: stress; Th1; Th2; autoimmune thyroid disease; Hashimoto thyroiditis; Graves' disease; glucocorticoids; catecholamines; epigenetics

9

Reactive intermediates, inflammation and epigenetics in lupus

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9.1 Introduction

The broad heterogeneous phenotype of systemic lupus erythematosus (SLE) is characterized by autoantibody production, a function primarily of the acquired immune response, leading to immune-complex deposition in target organs and resultant organ damage due to the release of inflammatory mediators [1]. An inappropriately active innate immune response, primarily via Toll-like receptor signalling, is implicated in both the initiation and pathogenic consequences of autoantibody production in SLE. An important arm of the innate immune response is the production of reactive intermediates, including the reactive oxygen intermediate (ROI) superoxide (SO; half-life ($t_{1/2}$) $\approx 10^{-9}$ s [2]) and the reactive nitrogen intermediates (RNIs) nitric oxide ($t_{1/2} \approx 3$–5 s [3]) peroxynitrite ($ONOO^-$; $t_{1/2} \approx 1$ s [4]) and hypochlorous acid (HOCl, $t_{1/2} \approx 30$ s [5,6]).

9.2 Biology of reactive intermediates

Reactive intermediates are short-lived molecules formed by chemical reactions and which are capable of rapidly modifying other molecules including nucleic acids, amino acids and lipids. Through these modifications they act as signalling molecules for a broad array of cellular functions. The pathogenic potential of NO is partly dependent upon whether its production occurs in proximity to the formation of ROIs such as SO.

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Depending on concentrations and biological setting, RNIs and ROIs can either neutralize each other or accentuate the activity of one another. Reactive intermediates are detoxified by protective enzymes such as superoxide dismutase (which catalyses the reaction of SO to H_2O_2). H_2O_2, not a free radical, is further catalysed in some cell types to HOCl (bleach) by myeloperoxidase (MPO) with an antimicrobial effect. H_2O_2 can also be converted to hydroxyl radicals (OH; $t_{1/2} \approx 10^{-9}$ s [7]) in the presence of Fe(II) via the Fenton reaction.

Nitric oxide

NO is a membrane-permeable free radical molecule synthesized by nitric oxide synthase (NOS) using arginine and oxygen as substrates [3]. Three isoforms of NOS are transcribed from three separate genes. All isoforms dimerize in the presence of co-factors to become active. Each monomer contains a reductase and oxygenase domain. The reductase domain catalyses the transfer of two electrons to haem iron in the oxygenase domain. Calmodulin, nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) are required co-factors for the reductase domain. Electrons from the reductase domain are transferred to the oxygenase domain of the adjacent monomer, where haem and tetrahydrobiopterin (BH_4) act as co-factors. Here, a reaction between O_2 and L-arginine is catalysed, resulting in formation of NO and citrulline.

Two isoforms (the endothelial NOS or eNOS/NOS3 gene product and neuronal NOS or nNOS/NOS1 gene product) are generally constitutively expressed and are dependent on sufficient concentrations of calcium for activity. In the vascular system, NO produced by eNOS is a potent vasodilator and regulator of vascular tone in response to shear stress. Nitroglycerin mimics the activity of eNOS by acting as a donor of NO [3]. Indigenous produced carriers of NO, including *S*-nitrosoglutathione, are capable of releasing NO in low concentrations also impacting biological function similar to direct eNOS NO release. The beneficial effect of NO produced by the constitutively expressed NOS isoforms is blunted when NO is produced in an environment high in ROI, as will be discussed later.

A third NOS gene (NOS2) produces an inducible isoform (iNOS) that is primarily expressed in immune cells, most notably macrophages and macrophage-derived cells. iNOS is expressed in response to specific inflammatory stimuli that are well characterized in murine cells, but the stimuli that induce NO in human cells are less well characterized. Among these stimuli for murine cells are several cytokines and Toll-like receptor ligands such as lipopolysaccharide, CpG DNA, interleukin-6 (IL-6), interferon-γ (IFN-γ), IL1-β and tumour necrosis factor α (TNF-α). In human cells, complex mixtures of cytokines are necessary for iNOS induction. In most cells, signalling pathways converge on the Janus kinase/signal transducer and activator of transcription (JAK/STAT) and/or the nuclear factor κB (NF-κB) pathways [8]. Nuclear hormone receptors also play a role in regulation of iNOS induction. There is evidence to support a role for oestrogen as an enhancer [9] and peroxisome proliferator-activated receptor γ (PPARγ) ligands as

inhibitors [10] of iNOS induction in response to IFN-γ or IFNγ- and lipopolysaccharide stimulation in murine cells. iNOS is expressed during pathological states in human endothelial cells, synovial fibroblasts, polymorphonuclear cells, lymphocytes and natural killer cells [11]. In normal human tissue, expression is also present in myocytes, skeletal muscle and Purkinje cells [12].

Overall, lupus can be described as a disease of heightened gene transcription, suggesting that it may result from inappropriate, somewhat global, transcriptional regulation. This altered transcriptional regulation may also contribute to overproduction of reactive intermediates. Work by Mishra *et al.* on histone deacetylase (HDAC) inhibitors best illustrates this concept [13,14]. Overactive or inappropriate HDAC activity leads to chromosomal conformational changes, allowing the transcriptional machinery access to genes for activation. Inhibitors of HDAC are currently approved for use in specific tumours, but are also very effective modulators of disease in murine models of lupus [13,14]. Indeed, HDAC inhibitors given either prior to disease onset or after disease onset have significant impacts on survival and renal disease development. NO production is also decreased with HDAC inhibitor therapy *in vivo*, but whether this is a primary or secondary effect remains to be determined. The HDAC inhibitor trichostatin A, which attenuated renal disease in MRL-*lpr/lpr* mice, inhibited NO production in cultured mesangial cells from the same murine model of lupus, suggesting that HDAC inhibitors probably act via direct effects on NO production, although other effects of HDAC inhibitors may also inhibit NO production indirectly [15]. Nonetheless, these data indicate that the epigenetic effects of histone acetylation can impact disease and production of reactive intermediates.

iNOS produces amounts of NO that are orders of magnitude higher than the constitutively expressed isoforms. In a low-arginine environment, iNOS cannot transfer nitrogen to molecular oxygen, and electrons from the reductase domain combine with oxygen to produce SO [16]. NO, when combined with SO, forms $ONOO^-$, a more reactive and toxic molecule than NO. $ONOO^-$ produced by immune cells is capable of killing intracellular pathogens and tumour cells. Glutathione peroxidase, catalase, superoxide dismutase, haem oxygenase and antioxidants serve to protect host cells during inflammatory states by reducing the total ROI burden that can contribute to $ONOO^-$ production [17,18]. Thus, reactive intermediate production depends on catalytic enzyme activity and substrate availability as well as the amount and activity of detoxifying enzymes (Figure 9.1).

The balance of production and elimination of reactive intermediates can be inferred by studying the modifications of target molecules that form from reactive intermediate production. Because their half-life is extremely short (albeit highly variable), reactive intermediates cannot be measured directly. NO and NO-derived products, however, can be measured by their ability to modify other molecules. There are three general ways by which these modifications occur. First, they can bind to metal centres. An example of this is guanylate cyclase, in which NO binds the ferrous state of the haem centre, induces a conformational change and increases the activity of the enzyme. The second modification involves S-nitrosylation. This often occurs at cysteine residues and has varying effects depending upon the target protein. For instance, caspase-3 activity is

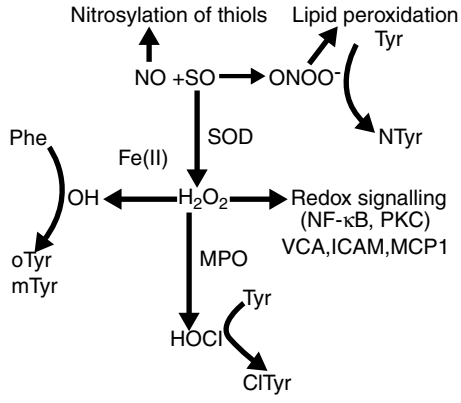

Figure 9.1 Simplified scheme illustrating the fate of NO and ROIs in biological systems. Note the ability of many reactive intermediates to modify amino acids to form modified tyrosine. ICAM, intercellular cell-adhesion molecule; MCP1, monocyte chemotactic protein-1; PKC, protein kinase C; VCAM, vascular cell adhesion molecule.

reduced by S-nitrosylation, while the activity of the ryanodine receptor increases with this modification, which is similar to the effect of other post-translational modifications on this protein, including phosphorylation, myristoylation and palmitoylation [19]. Another important product of nitrosylation is *S*-nitrosoglutathione, which can act as an intermediate leading to the glutathionylation of protein cysteines. This modification can also have functional significance [20]. The third modification from NO-derived products is a nitration reaction. Nitration generally occurs with higher oxides of nitrogen, typically $ONOO^-$. An example of this process is the formation of nitrotyrosine by $ONOO^-$ [4]. Nitration *in vitro* affects the activity of enzymes such as catalase and prostacyclin synthase [21]. In addition, nitrotyrosine modifications increase the antigenicity of proteins, thus acting as a potential mechanism for immune responses to self antigens [19].

Nitrosylation is being increasingly recognized as a mechanism for impacting gene regulation, similar to methylation and acetylation. Nitrosylation of NF-κB modulates its function, altering resultant inflammatory gene transcription. Such nitrosylation does not appear to impact the nuclear migration of NF-κB, but rather modulates its transcriptional activity once inside the nucleus [22]. Such nitrosylation can be achieved *in vivo* by administering *S*-nitrosoglutathione, providing another potential therapeutic pathway via modulation of reactive intermediates.

Measures of nitric oxide production

NO can react with oxygen in the presence of water to form nitrite, which can be further oxidized to nitrite and nitrate (NO_x). Nitrate is a stable oxidative product of NO and/or $ONOO^-$ in an aqueous environment and can be measured easily in biological fluids.

Modified tyrosine residues can serve as markers of HOCl (chlorotyrosine), $ONOO^-$ (nitrotyrosine) and OH (ortho- and metatyrosine production). These protein modifications are generally stable for the half-life of the protein with a few exceptions [19]. Thus, any measure of nitrosative or oxidative stress one chooses must be used with the knowledge that it reflects a balance between the production and removal of the reactive intermediates measured. Of the measures of NO production, the nitrosylation of tyrosine (producing 3-nitrotyrosine) appears the most reliable and reproducible. Reasons for this are that (1) the modifications are stable and thus reflect exposure to reactive intermediates for the life of the protein (allowing for a long-term indicator of reactive intermediate production analogous to glycosylated haemoglobin as a marker of glucose control), (2) these markers are not directly affected by diet and (3) the assay simultaneously detects markers of ROIs, RNIs and hypochlorous acid.

9.3 RNIs in murine models of lupus

Observational studies

Whereas iNOS activity is beneficial by suppressing parasitaemia and tumour growth, its overexpression in the setting of lupus leads to organ damage and an altered immune response. Both MRL/MpJ-*Fas*^{lpr}/J (MRL/lpr) and New Zealand Black × New Zealand White (NZB/W) F_1 mice develop spontaneous proliferative lupus nephritis. MRL/lpr mice had increasing levels of urine NO metabolites (nitrate and nitrite) in parallel with the onset of glomerulonephritis [23]. This increase in iNOS activity was associated with formation of 3-nitrotyrosine, a product of $ONOO^-$ and tyrosine. One such documented protein modification reduced the activity of catalase in the MRL/lpr kidney. As catalase removes superoxide, its inactivation exposes renal cells to increased oxidative stress and accelerated tissue damage [21].

Immune-complex formation and tissue deposition in lupus are not dependent on iNOS activity as iNOS inhibitor therapy, while improving renal histopathology, had no effect on glomerular immune-complex deposition in MRL/lpr mice [23]. The expression of iNOS appears to be a result of downstream innate immune responses to immune-complex deposition. A similar link between autoantibody deposition and iNOS expression/3-nitrotyrosine formation was observed in passive transfer models of anti-glomerular basement membrane and anti-MPO antibody-mediated glomerulonephritis [24–26].

Manipulation of inducible NOS in murine lupus

Several studies utilizing competitive inhibitors of iNOS suggest that iNOS activity is pathogenic in murine lupus. Inhibiting iNOS activity in MRL/lpr mice before disease onset with the non-specific arginine analogue L-N^G-monomethyl-L-arginine

(L-NMMA) reduced 3-nitrotyrosine formation in the kidney, partially restored renal catalase activity and inhibited cellular proliferation and necrosis within the glomerulus [21,23,24]. The partially selective iNOS inhibitor L-N^6-(1-iminoethyl) lysine (L-NIL) had a similar effect when used to treat these mice prior to disease onset. In this study, the L-NIL-treated mice exhibited significant improvement in glomerular histopathology compared to controls and slight improvements compared to L-NMMA-treated mice. L-NMMA therapy in NZB/W mice that were already suffering from nephritis had a similar but less profound effect on proteinuria and renal histopathology than did preventative therapy. However, L-NMMA as monotherapy for the treatment of active disease was less effective in the rapidly progressive MRL/lpr model [27].

Some interventions that do not directly inhibit iNOS enzyme activity may derive additional benefit by their ability to reduce expression of iNOS. For instance, chemical induction of haem oxygenase-1 and oral administration of mycophenolate mofetil were both effective therapies for treating glomerulonephritis in MRL/lpr mice, and both reduced iNOS expression in the kidney [23–25].

In contrast to the effectiveness of pharmacological iNOS inhibition in murine lupus is the observation that iNOS-deficient MRL/lpr mice, while having reduced signs of vasculitis, had similar glomerular pathology to their MRL/lpr wild-type littermates [27]. These findings may have several explanations. First, when iNOS is genetically absent, other compensatory mechanisms that are not activated in acute blockade by pharmacological inhibitors can induce renal damage. Second, the pharmacological inhibitors are not specific for iNOS, also blocking eNOS and/or nNOS, which may also play a role in disease (see below). Third, complete lack of iNOS, as present in the genetic knockouts, may not be as effective/desirable as partial inhibition achieved with pharmacological agents due to beneficial physiological functions of iNOS. We believe that all three explanations may be playing a role in the contrast between disease modulation by genetic versus pharmacological block of iNOS.

Potential mechanisms for pathogenicity of RNIs suggested by studies in murine models of lupus

The mechanisms through which iNOS activity may be pathogenic in SLE have been studied in animal models and *in vitro*. As mentioned above, $ONOO^-$, a byproduct of iNOS activity, can nitrate amino acids and change the catalytic activity of enzymes. One such enzyme, catalase, serves to protect host tissues from free-radical attack [21]. In vascular tissue, prostacyclin synthase [28] and eNOS [29] are inactivated by $ONOO^-$, leading to vasoconstriction. These observations suggest that one mechanism through which iNOS activity is pathogenic is via deactivation of tissue-protective enzymes.

Increasing attention is focusing on the manner in which immune tolerance is broken by presentation of autoantigens in a novel manner. Two such processes are

noteworthy: (1) presentation of nuclear antigens in the pro-inflammatory context of late apoptotic blebs and (2) post-translational modification of self-antigens to form novel epitopes or neoepitopes. Because nuclear antigens are present in late apoptotic blebs [30], regulation of apoptosis and clearance of apoptotic cells is an important area of investigation in the pathogenesis of lupus. Many alterations in apoptosis and debris-clearance pathways can lead to lupus-like disease as demonstrated in specific knockout mice (i.e. DNase and C4).

NO and $ONOO^-$ are both integral in regulating non-receptor-mediated apoptosis in many cellular systems [31]. To investigate the role of iNOS activity in apoptosis, MRL/lpr mice with active disease were treated with L-NMMA, an NOS inhibitor. Compared to controls, treated mice exhibited reduced levels of splenocyte apoptosis. Treatment of cultured splenocytes, isolated from mice with active disease, with an NO donor resulted in increased levels of apoptosis [32]. NO, or other RNIs, increased non-Fas receptor-mediated apoptosis despite the well-described defect in Fas receptor-mediated apoptosis in this murine model of lupus [32].

Another mechanism for inducing autoreactivity is via formation of neoepitopes in autoantigens. $ONOO^-$ can nitrate self-antigens in a manner that leads to a break in immune tolerance. For instance, normal mice immunized with nitrated IgGs produced anti-nitrotyrosine antibodies that cross-reacted with single-stranded DNA [33]. Human native DNA modified with $ONOO^-$ induced greater immunogenicity in experimental animals than native DNA without modifications [34].

RNIs in human SLE

Over the past decade several investigators have made important contributions to our understanding of the role of NO in SLE. Belmont *et al.* [35], Gonzalez-Crespo *et al.* [36], Wigand *et al.* [37], Rolla *et al.* [38] and our laboratory [39] have described increased markers of systemic NO production in SLE in a manner that parallel disease activity. However, it soon became clear [40] that those with lupus nephritis had the most elevated markers of systemic NO production among SLE subjects. This observation spawned the hypothesis that glomerular proliferative lesions were a source of increased NO production, as well as a potential result of inappropriate NO production. Wong *et al.* [41], Wang *et al.* [42] and our laboratory [40] supported this hypothesis with renal biopsy studies showing increased iNOS expression in the glomeruli of lupus nephritis subjects, particularly in mesangial cells, glomerular epithelial cells, and infiltrating inflammatory cells [42]. When 3-nitrotyrosine was used as a surrogate for iNOS activity, the association with disease activity was greater in African-Americans [40], suggesting a possible difference between Caucasians and African-Americans in ROI production compared with RNI production. While these studies focused on the kidney, Clancy *et al.* [43] and Belmont *et al.* [35] described an activated endothelial cell phenotype in lupus patients in which iNOS expression in endothelial cells was associated with a marker of systemic NO production.

Potential role of endothelial NOS in lupus

Nagy *et al.* [44] and Gergley *et al.* [45,46] published elegant studies demonstrating that abnormal T cell signal transduction in SLE is NO-mediated and dependent on Ca^{2+}. This calcium dependence suggested that a constitutive NOS isoform (cNOS) was required for NO production in lupus T cells. Further studies indicated that the basis of the abnormal T cell activation could be traced to mitochondrial hyperpolarization, which is dependent on mitochondrial NO production. Western blotting of peripheral blood lymphocyte lysates from SLE subjects revealed enhanced eNOS and nNOS expression, but not iNOS, compared to normal controls [44]. As NO appears to mediate mitochondrially mediated apoptosis and necrosis in SLE peripheral blood lymphocytes, NO may serve to increase the presentation of nuclear autoantigens in SLE. Thus, reactive oxygen/nitrogen intermediates probably play roles in both autoantibody production via the cNOS isoforms and tissue inflammation and endothelial cell activation after immune-complex deposition and complement via iNOS (Figure 9.2).

Mitochondrial hyperpolarization and T cell activation in SLE

Lymphocyte activation, proliferation and programmed cell death are dependent on controlled production of ROIs and ATP in mitochondria [47,48]. For the last few years, Perl *et al.* have focused their efforts on the role of mitochondria in T cell activation and death signal processing. They identified elevation of mitochondrial transmembrane potential ($\Delta\psi_m$); that is, mitochondrial hyperpolarization (MHP) and ATP depletion as an early and *reversible* step in normal T cell activation and apoptosis [46,49]. In contrast, they found that T lymphocytes of SLE patients exhibited *persistent* MHP, cytoplasmic alkalinization, increased ROI production and diminished levels of intracellular ATP and glutathione (GSH) [46]. These data clearly show that mitochondrial dysfunction leading to ATP depletion is ultimately

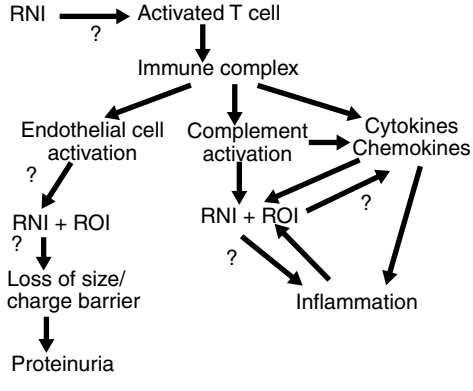

Figure 9.2 Postulated multifactorial role of reactive intermediates in the pathogenesis of lupus.

responsible for decreased activation-induced apoptosis which thus sensitizes lupus T cells for necrosis [46]. The resultant necrotic cell death would stimulate macrophages [50] and dendritic cells [51] leading to inflammation [46,48] and ultimate development of murine SLE [52]. Nagy *et al.* discovered that T cell activation-induced MHP was mediated through ROI-dependent expression of NOS isoforms eNOS and nNOS, which in turn required elevated Ca^{2+} levels for enzymic activity and NO production [44]. Persistent MHP was associated with increased mitochondrial mass, increased mitochondrial and cytoplasmic [Ca^{2+}] in T cells and enhanced NO production by monocytes in lupus patients. NO-induced mitochondrial biogenesis in normal T cells enhanced the rapid phase and reduced the plateau of Ca^{2+} influx upon CD3/CD28 co-stimulation, thus mimicking the Ca^{2+} signalling profile of lupus T cells.

Since mitochondria are major Ca^{2+} stores, NO-dependent mitochondrial biogenesis may account for altered Ca^{2+} handling by lupus T cells. MHP [46] and increased cytoplasmic [Ca^{2+}] are detectable in $CD4^+$ and $CD8^+$ and naive ($CD4^+CD45RA^+$) and memory ($CD4^+CD45RO^+$) T cell subsets [53,54], suggesting that mitochondrial dysfunction is a fundamental defect of most T cells in SLE patients. Indeed, MHP is also found in T cells of lupus-prone MRL/lpr, but not control $MRL^{+/+}$ mice. Therefore, future determination of the molecular basis of MHP is critically important for understanding the mechanism of abnormal T cell activation and death in human and murine SLE.

9.4 Genetic associations of RNI/ROI and lupus

Polymorphisms in NOS in human SLE

Based on studies demonstrating high NO production in lupus patients, we and others explored whether iNOS polymorphisms that enhance NO production are associated with development of lupus. In humans, NO production appears essential for elimination of specific pathogens (e.g. malaria parasites) [55]. We hypothesized that NOS2 polymorphisms associated with increased systemic NO production, which are protective against cerebral malaria [56], may confer an enhanced risk for the development of SLE. In African-American women with SLE, compared to race- and sex-matched controls from the Carolina Lupus cohort (CLu), the relative risk of developing SLE was 3.4 in those with the iNOS GC7343 polymorphism ($P = 0.04$) [57]. Analysis of this NOS2 polymorphism by other groups have also reported an association between lupus and this single nucleotide polymorphism, associated with high NO production.

Polymorphisms in NOS3 that are also associated with enhanced NO production were found associated with lupus by some groups, but not all. Most of these studies are small cohort studies and await confirmation by larger multi-ethnic cohort studies [58]. A recent study from Greece found an association between NOS3 polymorphisms and lupus nephritis [59]. These genetic studies further support the view that the effects of RNIs and ROIs on disease are significantly affected by the biological setting in which they are produced and the overall production of RNIs compared with ROIs.

ROI polymorphisms in lupus

To assess whether genes involved in ROI production are associated with lupus, our group studied polymorphisms in the gene for MPO. Surprisingly, there was a significant correlation between the low-expressing MPO 463A allele and the risk for developing lupus nephritis in African-Americans in the Carolina Lupus cohort [60]. This association was subsequently confirmed in two other cohorts. This finding may seem paradoxical until one considers that ROIs can sequester RNIs and that low MPO activity can lead to increased OH radical stress. To determine how this polymorphism may affect reactive intermediate production, serum was analysed for modified tyrosine, comparing those with the low-expressing A allele to those with G alleles. Those with the A/A genotype had significantly lower ratios of chlorotyrosine/orthotyrosine than those with G/A and G/G genotypes (1.5 ± 1.6 compared with 6.4 ± 11.1, $P = 0.04$). This finding is consistent with higher MPO activity in the G allele, for if MPO activity is high, chlorotyrosine levels should increase, whereas ortho- and metatyrosine formation should decrease. Thus, the A allele may have significant impact on the ROI/RNI spectrum produced during inflammation and may also impact disease expression.

9.5 Conclusions

In summary, this large and complex area of biology/medicine indicates that NO is overproduced in both human and murine lupus, as confirmed by a number of laboratories. Manipulating NO production pharmacologically, but not genetically, modulates disease expression in mice. Overproduction of NO can lead to disease pathogenesis through a number of different pathways including the more recent description of its impact on T cell function through MHP. NO, however, is not the only reactive intermediate overproduced in lupus. ROIs are also implicated in disease. cNOS isoforms may contribute to disease via effects on immune-cell activation and apoptosis. Further studies may reveal specific targets for therapeutic intervention in autoimmune diseases by modulating RNI or ROI production.

References

1. Hahn, B.H. (2002) An overview of the pathogenesis of systemic lupus erythematosus. In: Wallace D.J., Hahn B. and Dubois E.L. (eds), *Dubois' Lupus Erythematosus*, 6th edn, pp. 87–96. Lippincott Williams & Wilkins, Philadelphia.
2. Zahrt, T.C. and Deretic, V. (2002) Reactive nitrogen and oxygen intermediates and bacterial defenses: unusual adaptations in Mycobacterium tuberculosis. *Antioxidant Redox Signaling* **4**, 141–159.
3. Czapski, G. and Goldstein, S. (1995) The role of the reactions of NO with superoxide and oxygen in biological systems: a kinetic approach. *Free Radical Biology in Medicine* **19**, 785–794.
4. Liu, P., Xu, B., Quilley, J. and Wong, P.Y. (2000) Peroxynitrite attenuates hepatic ischemia-reperfusion injury. *American Journal of Physiology Cellular Physiology* **279**, C1970–C1977.

5. Pullar, J.M., Winterbourn, C.C. and Vissers, M.C. (1999) Loss of GSH and thiol enzymes in endothelial cells exposed to sublethal concentrations of hypochlorous acid. *American Journal of Physiology Heart and, Circulatory Physiology* **277**, H1505–H1512.
6. Oates, J.C. and Gilkeson, G.S. (2006) The biology of nitric oxide and other reactive intermediates in systemic lupus erythematosus. *Clinical Immunology* **121**, 243–250.
7. Yan, E.B., Unthank, J.K., Castillo-Melendez, M. *et al.* (2005) Novel method for in vivo hydroxyl radical measurement by microdialysis in fetal sheep brain in utero. *Journal of Applied Physiology* **98**, 2304–2310.
8. Kleinert, H., Pautz, A., Linker, K. *et al.* (2004) Regulation of the expression of inducible nitric oxide synthase. *European Journal of Pharmacology* **500**, 255–266.
9. Karpuzoglu, E. and Ahmed, S.A. (2006) Estrogen regulation of nitric oxide and inducible nitric oxide synthase (iNOS) in immune cells: Implications for immunity, autoimmune diseases, and apoptosis. *Nitric Oxide* **22**, 245–251.
10. Reilly, C.M., Oates, J.C., Sudian, J. *et al.* (2001) Prostaglandin J(2) inhibition of mesangial cell iNOS expression. *Clinical Immunology* **98**, 337–345.
11. Lincoln, J., Hoyle, C.H.V. and Burnstock, G. (1997) *Nitric Oxide in Health and Disease*, Cambridge University Press, New York.
12. Human Proteome Resource Program. *NOS2A in normal tissues*. www.hpr.se/tissue_profile.php?antibody_id=2014 (accessed 19 May 2006).
13. Mishra, N., Reilly, C.M., Brown, D.R. *et al.* (2003) Histone deacetylase inhibitors modulate renal disease in the MRL-lpr/lpr mouse. *Journal Clinical Investigation* **111**, 539–552.
14. Reilly, C.M., Mishra, N., Miller, J.M. *et al.* (2004) Modulation of renal disease in MRL/lpr mice by suberoylanilide hydroxamic acid. *Journal of Immunology* **173**, 4171–4178.
15. Garcia, B.A., Busby, S.A., Shabanowitz, J. *et al.* (2005) Resetting the epigenetic histone code in the MRL-lpr/lpr mouse model of lupus by histone deacetylase inhibition. *Journal of Proteome Research* **4**, 2032–2042.
16. Xia, Y. and Zweier, J.L. (1997) Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. *Proceedings of the National Academy of Sciences USA* **94**, 6954–6958.
17. Gaertner, S.A. Janssen, Ostendorf, T. *et al.* (2002) Glomerular oxidative and antioxidative systems in experimental mesangioproliferative glomerulonephritis. *Journal of the American Society of Nephrology* **13**, 2930–2937.
18. Abraham, N.G. and Kappas, A. (2005) Heme oxygenase and the cardiovascular-renal system. *Free Radical Biology in Medicine* **39**, 1–25.
19. Sato, E., Simpson, K.L., Grisham, M.B. *et al.* (1999) Effects of reactive oxygen and nitrogen metabolites on MCP-1-induced monocyte chemotactic activity in vitro. *American Journal of Physiology Lung Cellular & Molecular Physiology* **277**, L543–L549.
20. Hashemy, S.I. and Holmgren, A. (2008) Regulation of the catalytic activity and structure of human thioredoxin 1 via oxidation and S-nitrosylation of cysteine residues. *Journal of Biological Chemistry* **283**(32), 21890–21898.
21. Keng, T., Privalle, C.T., Gilkeson, G.S. *et al.* (2000) Peroxynitrite formation and decreased catalase activity in autoimmune MRL-lpr/lpr mice. *Molecular Medicine* **6**, 779–792.
22. Prasad, R., Giri, S., Nath, N. *et al.* (2007) GSNO attenuates EAE disease by S-nitrosylation-mediated modulation of endothelial-monocyte interactions. *Glia* **55**, 65–77.
23. Weinberg, J.B., Granger, D.L., Pisetsky, D.S. *et al.* (1994) The role of nitric oxide in the pathogenesis of spontaneous murine autoimmune disease: increased nitric oxide production and nitric oxide synthase expression in MRL-lpr/lpr mice, and reduction of spontaneous glomerulonephritis and arthritis by orally administered NG-monomethyl-L- arginine. *Journal of Experimental Medicine* **179**, 651–660.

24. Bremer, V., Tojo, A., Kimura, K. *et al.* (1997) Role of nitric oxide in rat nephrotoxic nephritis: comparison between inducible and constitutive nitric oxide synthase. *Journal of the American Society of Nephrology* **8**, 1712–1721.
25. Heeringa, P., van Goor, H., Moshage, H. *et al.* (1998) Expression of iNOS, eNOS, and peroxynitrite-modified proteins in experimental anti-myeloperoxidase associated crescentic glomerulonephritis. *Kidney International* **53**, 382–393.
26. Ohse, T., Ota, T., Kieran, N. *et al.* (2004) Modulation of interferon-induced genes by lipoxin analogue in anti-glomerular basement membrane nephritis. *Journal of the American Society of Nephrology* **15**, 919–927.
27. Oates, J.C., Ruiz, P., Alexander, A. *et al.* (1997) Effect of late modulation of nitric oxide production on murine lupus. *Clinical Immunology and Immunopathology* **83**, 86–92.
28. Zou, M.H., Cohen, R. and Ullrich, V. (2004) Peroxynitrite and vascular endothelial dysfunction in diabetes mellitus. *Endothelium* **11**, 89–97.
29. Zou, M.H., Shi, C. and Cohen, R.A. (2002) Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *Journal of Clinical Investigation* **109**, 817–826.
30. Casciola-Rosen, L.A., Anhalt, G. and Rosen, A. (1994) Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *Journal of Experimental Medicine* **179**, 1317–1330.
31. Boyd, C.S. and Cadenas, E. (2002) Nitric oxide and cell signaling pathways in mitochondrial-dependent apoptosis. *Biological Chemistry* **383**, 411–423.
32. Oates, J.C. and Gilkeson, G.S. (2004) Nitric oxide induces apoptosis in spleen lymphocytes from MRL/lpr mice. *Journal of Investigative Medicine* **52**, 62–71.
33. Ohmori, H., Oka, M., Nishikawa, N.Y. *et al.* (2005) Immunogenicity of autologous IgGs bearing the inflammation-associated marker 3-nitrotyrosine. *Immunology Letters* **96**, 47–54.
34. Habib, S., Moinuddin, M. and Ali, R. (2005) Peroxynitrite modified DNA: a better antigen for systemic lupus erythematosus anti-DNA autoantibodies. *Biotechnology and Applied Biochemistry* **45**, 265–271.
35. Belmont, H.M., Levartovsky, D., Goel, A. *et al.* (1997) Increased nitric oxide production accompanied by the up-regulation of inducible nitric oxide synthase in vascular endothelium from patients with systemic lupus erythematosus. *Arthritis and Rheumatism* **40**, 1810–1816.
36. Gonzalez-Crespo, M.R., Navarro, J.A., Arenas, J. *et al.* (1998) Prospective study of serum and urinary nitrate levels in patients with systemic lupus erythematosus. *British Journal of Rheumatology* **37**, 972–977.
37. Wigand, R., Meyer, J., Busse, R. and Hecker, M. (1997) Increased serum NG-hydroxy-L-arginine in patients with rheumatoid arthritis and systemic lupus erythematosus as an index of an increased nitric oxide synthase activity. *Annals of Rheumatic Diseases* **56**, 330–332.
38. Rolla, G., Brussino, L., Bertero, M.T. *et al.* (1997) Increased nitric oxide in exhaled air of patients with systemic lupus erythematosus. *Journal of Rheumatology* **24**, 1066–1071.
39. Gilkeson, G., Cannon, C., Oates, J. *et al.* (1999) Correlation of serum measures of nitric oxide production with lupus disease activity. *Journal of Rheumatology* **26**, 318–324.
40. Oates, J.C., Christensen, E.F., Reilly, C.M. *et al.* (1999) Prospective measure of serum 3-nitrotyrosine levels in systemic lupus erythematosus: correlation with disease activity. *Proceedings of the American Association of Physicians* **111**, 611–621.
41. Wong, C.K., Ho, C.Y., Li, E.K. *et al.* (2002) Elevated production of interleukin-18 is associated with renal disease in patients with systemic lupus erythematosus. *Clinical and Experimental Immunology* **130**, 345–351.

42. Wang, J.S., Tseng, H.H., Shih, D.F. *et al.* (1997) Expression of inducible nitric oxide synthase and apoptosis in human lupus nephritis. *Nephron* **77**, 404–411.
43. Clancy, R., Marder, G., Martin, V. *et al.* (2001) Circulating activated endothelial cells in systemic lupus erythematosus: further evidence for diffuse vasculopathy. *Arthritis and Rheumatism* **44**, 1203–1208.
44. Nagy, G., Koncz, A. and Perl, A. (2003) T cell activation-induced mitochondrial hyperpolarization is mediated by Ca2 + - and redox-dependent production of nitric oxide. *Journal of Immunology* **171**, 5188–5197.
45. Gergely, P., Jr, Niland, B., Gonchoroff, N. *et al.* (2002) Persistent mitochondrial hyperpolarization, increased reactive oxygen intermediate production, and cytoplasmic alkalinization characterize altered IL-10 signaling in patients with systemic lupus erythematosus. *Journal of Immunology* **169**, 1092–1101.
46. Gergely, P., Jr, Grossman, C., Niland, B. *et al.* (2002) Mitochondrial hyperpolarization and ATP depletion in patients with systemic lupus erythematosus. *Arthritis and Rheumatism* **46**, 175–190.
47. Perl, A., Gergely, P., Jr, Nagy, G. *et al.* (2004) Mitochondrial hyperpolarization: a checkpoint of T-cell life, death and autoimmunity. *Trends in Immunology* **25**, 360–367.
48. Banki, K., Hutter, E., Gonchoroff, N.J. and Perl, A. (1999) Elevation of mitochondrial transmembrane potential and reactive oxygen intermediate levels are early events and occur independently from activation of caspases in Fas signaling. *Journal of Immunology* **162**, 1466–1479.
49. Nagy, G., Barcza, M., Gonchoroff, N. *et al.* (2004) Nitric oxide-dependent mitochondrial biogenesis generates Ca2 + signaling profile of lupus T cells. *Journal of Immunology* **173**, 3676–3683.
50. Cohen, J.J., Duke, R.C., Fadok, V.A. and Sellins, K.S. (1992) Apoptosis and programmed cell death in immunity. *Annual Reviews of Immunology* **10**, 267–293.
51. Sauter, B., Albert, M.L., Francisco, L. *et al.* (2000) Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *Journal of Experimental Medicine* **191**, 423–434.
52. Ma, L., Chan, K.W., Trendell-Smith, N.J. *et al.* (2005) Systemic autoimmune disease induced by dendritic cells that have captured necrotic but not apoptotic cells in susceptible mouse strains. *European Journal of Immunology* **35**, 3364–3375.
53. Vassilopoulos, D., Kovacs, B. and Tsokos, G.C. (1995) TCR/CD3 complex-mediated signal transduction pathway in T cells and T cell lines from patients with systemic lupus erythematosus. *Journal of Immunology* **155**, 2269–2281.
54. Kyttaris, V.C. and Tsokos, G.C. (2004) T lymphocytes in systemic lupus erythematosus: an update. *Current Opinion in Rheumatology* **16**, 548–552.
55. Anstey, N.M., Weinberg, J.B., Hassanali, M.Y. *et al.* (1996) Nitric oxide in Tanzanian children with malaria: inverse relationship between malaria severity and nitric oxide production/nitric oxide synthase type 2 expression. *Journal of Experimental Medicine* **184**, 557–567.
56. Kun, J.F., Mordmuller, B., Perkins, D.J. *et al.* (2001) Nitric oxide synthase 2 (G-954C), increased nitric oxide production, and protection against malaria. *Journal of Infectious Diseases* **184**, 330–336.
57. Oates, J.C., Levesque, M.C., Hobbs, M.R. *et al.* (2003) Nitric oxide synthase 2 promoter polymorphisms and systemic lupus erythematosus in African-Americans. *Journal of Rheumatology* **30**, 60–67.
58. Douglas, G., Reilly, C., Dooley, M.A. *et al.* Angiotensin-converting enzyme (insertion/deletion) and endothelial nitric oxide synthase polymorphisms in patients with systemic lupus erythematosus. *Journal of Rheumatology* **31**, 1756–1762.

59. Vazgiourakis, V., Sidiropoulos, P., Bertsias, G. *et al.* (2007) Association of the nitric oxide synthase (eNOS) gene polymorphism with increased risk for both lupus glomerulonephritis and rheumatoid arthritis in a single genetically homogeneous population. *Lupus* **16**, 867–874.
60. Bouali, H., Nietert, P., Nowling, T.M. *et al.* (2007) Association of the G-463A myeloperoxidase gene polymorphism with renal disease in African Americans with systemic lupus erythematosus. *Journal of Rheumatology* **34**, 2028–2034.

Abstract

Reactive intermediates include reactive nitrogen intermediates and reactive oxygen intermediates. They are primarily products of the innate immune system with cytotoxic properties towards microbial organisms and malignant cells. Reactive intermediates are also critical components of cell signalling pathways and induce a variety of physiological functions including vasodilatation and modulation of cell function. Reactive intermediates can interact with proteins, nucleic acids and lipids, altering their structure and function, including altering gene transcription. The end result of reactive intermediate production is highly dependent on the biological setting in which they are produced with the same molecule resulting in maintenance of important physiological functions or induction/accentuation of autoimmune disease and organ damage. Reactive intermediates are known to be overproduced in certain autoimmune diseases, including lupus, and play an important role in the pathogenesis of this disease. This review focuses on the role of reactive intermediates in organ damage and T cell function in lupus and the associations with polymorphisms in genes involved in reactive intermediate production.

Key words: reactive intermediates; nitric oxide; lupus; T cell function; murine models; glomerulonephritis

10

Post-translational modification of HMGB1 and its role in immune activation

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10.1 Introduction

High-mobility group box 1 (HMGB1) is a non-histone nuclear protein with dual function. Inside the cell HMGB1 interacts with DNA to modify its structure and regulate transcription. Outside the cell HMGB1 serves as an alarmin to activate the innate immune system and promote tissue repair. The translocation of HMGB1 from inside to outside the cell is essential to the immunological activity of this protein and takes place primarily during cell activation and cell death. In these settings, HMGB1 may undergo post-translational modifications similar to those determining the epigenome. Whereas usual epigenetic modifications influence gene expression, with HMGB1 these modifications influence the location of the protein in the cell as well as its immunological activity.

As described elsewhere in this volume, epigenetic modification of DNA and histones is a ubiquitous process regulating gene expression for differentiated cell function. These modifications represent a level of genetic information that extends beyond that encoded in the DNA itself. Although this information is distinct from the genome, it is nevertheless heritable [1,2]. The most prominent epigenetic modifications are CpG methylation of DNA and covalent modification of histones. Indeed, over 100 different post-translational protein modifications like methylation, phosphorylation, acetylation, ribosylation and ubiquitination characterize the epigenome [3,4].

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In addition to histones, other nuclear proteins may undergo post-translation modifications that impact their function. Among these proteins, HMGB1 is an abundant constituent of the nucleus, constituting an architectural element that can regulate gene transcription and DNA repair [5,6]. Whereas the post-translational modification of HMGB1 could have epigenetic effects by altering DNA interactions, a change in location of this protein provides another, unexpected mechanism by which protein modification can generate regulatory interactions important for intracellular as well as intercellular gene expression. This review will focus on the role of HMGB1 as an immunological mediator and its potential as a target of therapy that involves not only its extracellular activity but also its intracellular modification and translocation.

10.2 Molecular biology of HMGB1

The HMGB1 protein was initially discovered as a non-histone nuclear protein that stabilizes chromatin structure [7]. Along with two other proteins, HMGB2 and HMGB3, HMGB1 is a member of a family of proteins named on the basis of their mobility in gel electrophoresis. These nuclear proteins have highly conserved structures, displaying three different domains. Two domains of up to 80 amino acids form the homologous high-mobility group (HMG) boxes called A and B; these boxes contain three positively charged α-helices and bind to the minor groove of DNA. A highly negatively charged stretch of predominantly aspartate and glutamate residues forms the C-terminal domain, which is about 30 amino acids long [8,9]. The amphipathic nature of HMGB1 led to its designation as amphoterin [10].

HMGB1 is a 215-residue protein typically expressed in nucleated cell types and is only 10 times less abundant than the core histones. HMGB1 is evolutionarily conserved and shows high homology among mammals, with only two of the 215 residues substituted in the rodent and human versions [11–14]. Liver and brain cells have higher concentrations of HMGB1 in the cytoplasm than in the nucleus compared to other tissues whereas lymphoid tissues and testes have the highest nuclear concentrations [15].

In the nucleus, HMGB1 functions as a DNA-binding protein that associates with the DNA to modulate chromatin structure and promote the binding of transcription factors and chromatin-remodelling complexes [16–19]. DNA binding is dependent on both the A and B box, the former showing stronger affinity for distorted DNA structures [17,20]. The binding to distorted DNA can enhance the formation and sliding of nucleosome complexes by stabilizing these structures [21]. HMGB1 binds to the minor groove of DNA to distort the double helix and to allow interactions with transcription factors like nuclear factor κB (NF-κB), p53, steroid hormone receptors, homeobox-containing proteins and RAG1 recombinase [22–25]. HMGB1 gene-deficient mice die shortly after birth because of hypoglycaemia due to defective glucocorticoid receptor gene activation [26]. Because of its low affinity for DNA, HMGB1 appears to be very mobile within the nucleus and can translocate between the nucleus and the cytosol through nuclear pores [16,27,28].

10.3 HMGB1 as an immune mediator

Alarmin activity of HMGB1

The molecular properties of HMGB1 are consistent with its role in chromosomal architecture and transcriptional activity. Its immunological properties, however, were a great surprise, their discovery creating a new paradigm for activation of innate immunity by endogenous macromolecules. This discovery resulted from efforts to define new mediators of sepsis that could serve as more effective targets for therapy than cytokines such as tumour necrosis factor α (TNF-α) and interleukin (IL)-1 [29]. Although such cytokines play an important role in sepsis, their production is transient, with efforts at using anti-cytokine agents in human trials unsuccessful despite impressive results in animal models.

By characterizing proteins whose *in vitro* expression was induced by lipopolysaccharide (LPS), Wang *et al.* demonstrated that HMGB1 is released extracellularly by macrophages after stimulation with endotoxin [29]. The kinetics of this release differed from that of cytokines although a role of HMGB1 in sepsis in animals could be established by the effects of anti-HMGB1 antibodies as well as other blocking strategies. Importantly, in sepsis, HMGB1 showed more sustained production and functioned as a late mediator of septic shock in mice. In shock situations in humans, HMGB1 levels are elevated in the blood, suggesting that, in human disease as well as animal models, HMGB1 can promote pathogenesis. This conclusion is supported by observations that HMGB1 can induce inflammation when administered via intraperitoneal, intratracheal, intra-articular or intracerebroventricular routes [30].

As shown in experiments using cloned or purified preparations, HMGB1 displays a wide range of immunological activities that lead to its characterization as an alarmin. According to current terminology, an alarmin is a cellular macromolecule that can activate the innate immune system and promote chemotaxis. Alarmins can be released from injured or dying cells although some alarmins such as HMGB1 can originate from activated cells by non-traditional secretory mechanisms. Other examples of alarmins are IL-1α, hepatoma-derived growth factor (HDGF), defensins, cathelicidins, S100 proteins and heat-shock proteins, among others [31].

Because of its origin and immunological properties, HMGB1 can also be designated as a damage- (or death-) associated molecular pattern (DAMP) by analogy to a pathogen-associated molecular pattern, or PAMP [31]. Whereas PAMPs are exogenous molecules, DAMPs are endogenous molecules. Both DAMPs and PAMPs interact with cellular receptors such as the Toll-like receptors (TLRs) and induce similar downstream signalling events and responses, both physiological and pathological. HMGB1 can also interact with the receptor for advanced glycation end products (RAGE) [32,33]. Table 10.1 lists major immunological activities of HMGB1.

For HMGB1, immunological activity depends on exposure to cells of the innate immune system and therefore on its translocation or release from cells. This release occurs in the settings of activation and cell death, with release during death central to its role as an alarmin (Figure 10.1). While activation and cell death are seemingly opposite

Table 10.1 Immunological activities of HMGB1.

Cell/tissue targets of HMGB1	Activity
Monocytes/macrophages	Stimulate cytokine release (TNF, MIP-1α/1β, IL-1α/β, IL-6, IL-8)
Microvascular endothelial cells	Increase expression of cell surface markers (ICAM-1, RAGE, VCAM-1); enhance expression of TNF-α, IL-8, MCP-1, tPA, PAI-1
Neutrophils	Recruitment; activation of p38 MAPK, Akt and ERK1/2; cytokine production
Dendritic cells	Upregulate surface markers; enhance cytokine production
T lymphocytes	Proliferation
B lymphocytes	Promote VDJ recombination
Mesangioblasts	Recruitment to sites of tissue injury; promote repair
Smooth muscle cells, cardiac myocytes, osteoblasts, osteoclasts	Recruitment to sites of tissue injury
Astrocytes, neurones	Promote neurite outgrowths

ERK1/2, extracellular-signal-regulated kinase 1/2; ICAM-1, intercellular cell-adhesion molecule 1; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; MIP-1α/1β, macrophage inflammatory protein 1α/1β; PAI-1, plasminogen activator inhibitor 1; tPA, tissue plasminogen activator; VCAM-1, vascular cell adhesion molecule 1.

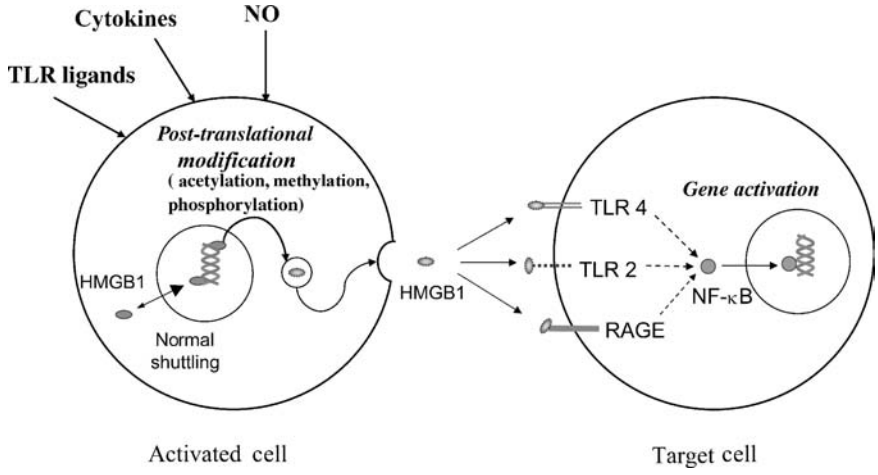

Figure 10.1 The role of post-translational modification in translocation and immune activation by HMGB1. HMGB1 undergoes post-translational modification (e.g., acetylation, phosphorylation, methylation, etc.) following cell activation induced by external stimuli. This modification leads to translocation of HMGB1 from the nucleus into the cytoplasm, into secretory endosomes and out of the cell. Extracellular HMGB1 functions as an immune activator by binding TLRs 2 and 4 and RAGE on immune cells like macrophages and neutrophils. Following binding, it leads to activation of gene expression via NF-κB.

fates of a cell, in the immune system these events are linked because of activation-induced cell death. Studies *in vitro* with a variety of cell systems have identified several distinct mechanisms of HMGB1 release from cells that, importantly, may involve post-translational modifications that can change the charge of HMGB1 as well as its interaction with chromatin. As such, these modifications resemble epigenetic modifications that occur on histones during similar events.

10.4 Mechanisms of HMGB1 modification and release

HMGB1 release during activation

The extracellular release of HMGB1 was first described in the context of activation by LPS, with this agent inducing the translocation of HMGB1 from the nucleus to the cytoplasm into secretory endosomes and eventual secretion [34,35]. Among other TLR ligands, polyinosinic:polycytidylic acid (poly(I:C)), a ligand for TLR3, can induce HMGB1 release from macrophages whereas CpG DNA, a ligand for TLR9, does not [36]. These findings suggest that activation by some but not all PAMPs causes changes that lead to HMGB1 translocation. Among other pro-inflammatory mediators, interferon (IFN)-γ, IFN-α/β, TNF-α and nitric oxide can all induce HMGB1 release from macrophages, suggesting that HMGB1 is part of a regulatory loop governing inflammation.

As shown in elegant molecular and cell biology studies, the translocation of HMGB1 depends upon protein modification, with acetylation playing a key role [34,35]. Thus, acetylation of Lys-2 and Lys-11 in the A-box of HMGB1 reduces binding to DNA and interaction with chromatin [20]. HMGB1 lacks a signal peptide, however, to determine its movement to the endoplasmic reticulum–Golgi system. Acetylation of lysine residues in two specific clusters in the protein, however, appears important for the movement of HMGB1 to the cytoplasm and blocking reentry to the nucleus as observed in activated monocytes [35]. The cytoplasmic pool of HMGB1 may then enter cytoplasmic organelles that undergo exocytosis under the influence of lysophosphatidylcholine, a product of inflammatory cell activation [34].

As in the case of histones, other protein modifications of HMGB1 may affect the DNA-binding properties of HMGB1 in different cell types. Thus, in activated murine macrophages, HMGB1 may be phosphorylated in the nuclear-localization signals at serine residues whereas, in activated neutrophils, methylation of HMGB1 occurs at Lys-42 [37,38]. Similarly, reversible protein S-glutathionylation of Cys-23, -45 and -106 residues induces conformational changes in HMGB1 in response to oxidative stress causing nucleocytoplasmic translocation and consequent functional changes [39]. As a result of these changes, which occur during activation, the location of HMGB1 changes and HMGB1 shows a different distribution between the cytoplasm and nucleus. While translocation of HMGB1 during cell activation is necessary for alarmin activity, its consequences for gene expression are not well understood. In view of the role of

HMGB1 in transcriptional regulation, a cell in which this protein has left the nucleus at least transiently could display altered patterns of gene expression, reflecting the change in chromosomal architecture.

Release of HMGB1 during necrosis

The release of HMGB1 is a prominent feature of cell death in addition to macrophage activation. In simplest form, cell death occurs in two main types: apoptosis and necrosis. Apoptosis, or programmed cell death, is a highly regulated process mediated by a cascade of enzymes called caspases that cause the systematic rearrangement, compaction and disassembly of cells. Apoptosis can occur in both physiological and pathological settings and be induced by a wide variety of triggers. In contrast, necrosis is a sudden and random form of cell death that is induced by physical or chemical trauma and leads to cell lysis. Necrosis is virtually always pathological.

In addition to their mechanisms, apoptosis and necrosis may differ in their immunological consequences. Thus, apoptotic death is usually considered to be non-inflammatory whereas necrotic cell death is considered to be pro-inflammatory. An important focus of research on the immunology of cell death has thus involved a search for molecules from apoptotic cells that block inflammation on the one hand and molecules from necrotic cells that induce inflammation on the other. Pro-inflammatory molecules from necrotic cells would represent DAMPs; at present there is no term for anti-inflammatory molecules from apoptotic cells.

Following discovery of HMGB1 as a mediator of inflammation released from activated cells, a series of seminal studies established its role as a pro-inflammatory mediator during necrotic cell death [28,40]. Depending on the experimental system for inducing necrosis, the release of HMGB1 occurs readily since HMGB1 is not tightly bound to chromatin. It can therefore readily exit from cells as permeability barriers break down or as the cell lyses during necrosis. This release process appears to be passive and rapid, especially when necrosis is induced by destructive treatments such as cycles of freezing and thawing.

In other settings of necrotic cell death, HMGB1 release may entail additional steps if necrosis is induced by a chemotherapeutic agent. While alkylating agents are usually thought to induce apoptosis, they can induce death with features of necrosis in cells that are deficient in certain regulators of apoptosis such as Bax and Bak. In such cells, treatment with DNA-alkylating agents leads to activation of poly(ADP)-ribose polymerase (PARP) which in turn regulates HMGB1 translocation from the nucleus to the cytoplasm and eventual leakage out of the damaged cell membrane [41]. This translocation requires the glutamate-rich C-terminal tail of HMGB1 which, however, does not appear to be the sole substrate for PARP modification. In PARP-deficient cells treated with an alkylating agent, HMGB1 appears to remain bound to chromatin and does not stimulate inflammation. Thus, during necrosis, HMGB1 may involve post-translational modification although, in badly damaged or lysed cells, diffusion alone may account for its release. Once outside the cell, HMGB1 can display alarmin activity and, indeed, appears to be a major source of the immunological activity of necrotic cells [40].

Localization of HMGB1 in apoptosis

The localization of HMGB1 during apoptotic death is variable. The original studies on this issue indicated that HMGB1 was not released from cells during apoptosis and appeared to stay anchored in the nucleus during this process. Furthermore, studies using fluorescence loss in photobleaching (FLIP) showed that HMGB1 has dramatically decreased intranuclear mobility during apoptosis [28]. This observation suggests a fundamental change in the interaction of HMGB1 with chromatin since, under usual situations, HMGB1 binds chromatin weakly and has free mobility in the nucleus. Evidence that post-translational modification leads to this change comes from experiments in which apoptotic cells were treated with trichostatin A, an inhibitor of histone deacetylase. With apoptotic cells treated with trichostatin A, HMGB1 release occurs during apoptosis, suggesting that an alteration of the structure of HMGB1 or histones can determine the interaction and localization of HMGB1 with chromatin during apoptosis.

The retention of HMGB1 in the nucleus of apoptotic cells could have immunological significance. If this protein is a major source of immunological activity of dead cells, its intracellular retention during apoptosis could diminish a potent stimulus for inflammation and allow the anti-inflammatory activities of apoptotic cells to dominate. Together, these studies led to an appealing and popular model in which HMGB1 localization represents an important switch governing the immunological activity of dead cells and their potential activity in diseases such as malignancy and sepsis where large numbers of cells die.

Subsequent experiments using other systems indicated that dichotomy in the behaviour of HMGB1 during death is not as stark as initially proposed. Thus, studies by Bell *et al.* showed that induction of apoptosis in Jurkat T cell leukaemia cells led to the release of HMGB1 as shown by Western blotting of the cell-culture media or confocal microscopy [42]. This release was observed with chemical inducers such as staurosporine as well as ultraviolet light and occurred at a late stage of apoptosis that can be called secondary necrosis. During this stage the permeability of the cell breaks down and other nuclear molecules such as DNA and histones are either translocated in the cell or released. Since HMGB1 binds to chromatin, simultaneous release would not be unexpected.

The differences in the behaviour of HMGB1 during apoptosis may reflect differences in the experimental systems such as the cell lines and inducers of apoptosis studied or the time course of the experiments. Furthermore, differences may relate to the manner in which necrosis is induced and the extent of cell damage that result. Treatments such as freeze-thawing can cause massive cell damage and abundant release of molecules, including HMGB1. In contrast, the amounts of HMGB1 released during apoptosis may be much less by comparison, despite being significant immunologically.

Although HMGB1 movement during apoptosis requires future investigation, clarification of this issue is important since it impacts on the interpretation of the immunological properties of apoptotic and necrotic cells. If both apoptotic and necrotic cells release HMGB1, differences in immunological activity may relate to the

ensemble of molecules released as opposed to the presence of a single molecule. Post-translational modification could also contribute to differences in the activity of HMGB1 observed in various settings, especially if changes such as acetylation or phosphorylation influence activity.

In the disease setting, the recognition that apoptotic cells release HMGB1 is important to conceptualizing the mechanisms of inflammation. Thus, the presence of extracellular HMGB1 may indicate apoptotic as well as necrotic cell death as well as inflammation. In skin lesions of systemic lupus erythematosus, for example, extracellular HMGB1 appears at sites of disease activity [43]. Rather than secretion from inflammatory cells in the lesion, this material could result from release from apoptotic cells. Similarly, in sepsis, extracellular HMGB1 may be the direct product of apoptotic cells rather than the consequence of an indirect process in which HMGB1 from necrotic cells causes activation of macrophages, which then secrete HMGB1 [44].

The setting of apoptosis is notable in the context of epigenetics. Thus, during the course of this process phosphorylation of histone H1 occurs prominently [45]. The basis for this change is not clear since apoptosis leads to death as opposed to activation or other cellular states in which epigenetic changes can modify patterns of gene expression. This consideration could suggest that epigenetic modification of histones is important in regulating transcription of genes crucial for apoptosis. Alternatively, histone modification during apoptosis could be part of a stress response that ultimately fails and the cell undergoes apoptosis. While post-translational modification of HMGB1 has not been studied in the context of apoptosis, the similarity between H1 and HMGB1 in terms of their DNA interactions suggests that HMGB1 may also be modified during death as well as activation.

While mechanisms for HMGB1 during macrophage activation and cell death appear distinct, there are nevertheless potential similarities. As noted above, among the TLRs studied, stimulation of TLR3 by poly(I:C) and TLR4 by LPS lead to HMGB1 release whereas stimulation of TLR9 by CpG DNA does not cause this release, despite cytokine induction [36,46]. It is of interest therefore that stimulation of TLR3 and TLR4 differs from that of TLR9 in both the involvement of the downstream adaptor protein Toll/IL-1 receptor (TIR)-domain-containing adaptor-inducing IFN-β (TRIF) pathway as well as the induction of apoptosis. Thus, both poly(I:C) and LPS induce much higher levels of apoptosis in cultures of murine macrophages than does CpG DNA [46]. Since apoptotic cells can release HMGB1, it is possible that at least some of the extracellular HMGB1 derives from apoptotic cells in the stimulated cultures.

10.5 The role of HMGB1 as a mediator of disease and target of therapy

HMGB1 has widespread activities in the immune system and can induce a host of responses such as cell proliferation, cytokine production and increased expression of cell-surface molecules involved in inflammation. These activities resemble those of

cytokines such as TNF-α and IL-1. Evidence that this protein mediates disease is extensive and derives from two main sources: (1) demonstration of extracellular HMGB1 or cellularly translocated HMGB1 in tissue or the blood from either animal models or patients with disease and (2) demonstration that blockade of HMGB1 ameliorates disease in animal models. While the role of HMGB1 was originally demonstrated in sepsis, studies have now implicated this protein in rheumatoid arthritis, systemic lupus erythematosus, Sjögren's syndrome and stroke among many others [43,47–52]. The list will undoubtedly grow and it is likely that HMGB1 contributes to the pathogenesis of any disease in which immune-cell activation or cell death occurs.

Among diseases where HMGB1 may promote pathogenesis, systemic lupus erythematosus is characterized by abnormalities in the extent of apoptotic death as well as impairment in the clearance of apoptotic cells. In this disease, immune complexes comprised of nuclear macromolecules and anti-nuclear antibodies form and deposit in the tissue to incite local inflammation. In addition, these complexes can stimulate the production of interferon α/β by plasmacytoid dendritic cells, a response that depends upon TLR9 as well as the Fcγ receptor IIa [53,54]. Furthermore, this response may involve HMGB1, which serves as a component of these complexes and stimulates responses via RAGE. HMGB1 may also promote responses to DNA in complexes by its interaction with TLR9 [55].

For these pathogenic complexes to form, both HMGB1 and nucleosomal antigens must translocate from the inside to the outside of cells. As discussed above, this translocation can occur during apoptosis as part of the rearrangement of molecules during this process as well as breakdown of cell permeability. At least some of this rearrangement may involve post-translational modification implicated in epigenetic regulation. In the case of complexes, HMGB1 and chromatin may exit cells bound together or interact in the circulation after their separate exit from cells. Since HMGB1 binds weakly to chromatin, formation of complexes in the circulation appears less likely to occur than in the cell where the concentrations of reactant would be higher. In this scenario, the exit of nuclear molecules from the cell is perhaps the most drastic consequence of epigenetic modification, impacting on gene expression of other cells by movement of these molecules.

At present, for the various diseases where HMGB1 may be pathogenic, strategies to block the effects of HMGB1 focus on the antibodies or other agents that bind to HMGB1 and therefore prevent its interaction with its receptors [44,56–59]. While both TLR2 and TLR4 can serve as receptors for HMGB1, RAGE appears to play a major role in the response to this protein [33,55,60]. Antibodies to RAGE have been used widely to treat inflammation in animal models, with their efficacy potentially including blocking of HMGB1-receptor interactions. Interestingly, an isolated domain of HMGB1 can block the effect of the intact protein. In animal models of collagen-induced arthritis, an A-box construct can attenuate disease [61].

These therapies target HMGB1 after it has left the cell. From the nature of this system, however, strategies to target the release of HMGB1 should also be possible. These strategies would include inhibitors of post-translational modification although

their effects may be broader if the same enzymes modifying HMGB1 also modify histones. An alternative approach is suggested by the effects of gold salts, a group of compounds that were once the mainstay of the treatment of rheumatoid arthritis. As shown in a study of murine macrophages *in vitro*, gold thiomalate (Myochrysine) can block the release of HMGB1 from murine macrophages stimulated by LPS. This effect was specific because gold thiomalate did not affect the release of TNF-α from these cells [62].

Whereas gold has other immune effects, it is possible that its anti-rheumatic activities results from interdiction of events important in the modification or translocation of HMGB1 during activation or a subsequent step in the intracellular trafficking. Platinum compounds can also block HMGB1 release from macrophages and are effective in animal models of arthritis [63]. These compounds can chemically modify DNA and create DNA adducts that avidly bind HMGB1. While the mechanisms of action of gold and platinum require further investigation, the results of these studies suggest a potential new target for the therapy of inflammatory and autoimmune disease.

10.6 Conclusion

HMGB1 is a non-histone nuclear protein that can activate the innate immune system and play a role in the pathogenesis of many inflammatory diseases. Similar to the situation with histones, HMGB1 can undergo post-translational modifications that affect its interaction with chromatin. These modifications can also affect the location of HMGB1 and its ability to display immunological activity. Future work will determine the mechanisms for these modifications, their impact on immunological activity and their potential as targets of new therapy.

References

1. Wolffe, A.P. and Matzke, M.A. (1999) Epigenetics: regulation through repression. *Science* **286**, 481–486.
2. Nakao, M. (2001) Epigenetics: interaction of DNA methylation and chromatin. *Gene* **278**, 25–31.
3. Bernstein, B.E., Meissner, A. and Lander, E.S. (2007) The mammalian epigenome. *Cell* **128**, 669–681.
4. Kouzarides, T. (2007) Chromatin modifications and their function. *Cell* **128**, 693–705.
5. Grosschedle, R., Giese, K. and Pagel, J. (1994) HMG domain proteins: architectural elements in the assembly of nucleoprotein structures. *Trends in Genetics* **10**, 94–100.
6. Reeves, R. and Adair, J.E. (2005) Role of high mobility group (HMG) chromatin proteins in DNA repair. *DNA Repair* **4**, 926–938.
7. Goodwin, G.H., Sanders, C. and Johns, E.W. (1973) A new group of chromatin-associated proteins with a high content of acidic and basic amino acids. *European Journal of Biochemistry* **38**, 14–17.
8. Bianchi, M.E., Falciola, L., Ferrari, S. *et al.* (1992) The DNA binding site of HMG1 proteins is composed of two similar segments (HMG boxes), both of which have counterparts in other eukaryotic regulatory proteins. *EMBO Journal* **11**, 1055–1063.

9. Bianchi, M.E. and Beltrame, M. (2000) Upwardly mobile proteins. The role of HMG proteins in chromatin structure, gene expression and neoplasia. *EMBO Reports* **1**, 109–119.
10. Parkkinen, J., Raulo, E., Merenmies, J. *et al.* (1993) Amphoterin, the 30-kDa protein in a family of HMG1-type polypeptides. *Journal of Biological Chemistry* **268**, 19726–19738.
11. Levy, W.B., Wong, N.C. and Dixon, G.H. (1977) Selective association of the trout-specific H6 protein with chromatin regions susceptible to Dnase I and Dnase II: possible location of HMG-T in the spacer region between core nucleosomes. *Proceedings of the National Academy of Sciences, USA* **74**, 2810–2814.
12. Paonessa, G., Frank, R. and Cortese, R. (1987) Nucleotide sequence of rat liver HMG1 cDNA. *Nucleic Acids Research* **15**, 9077.
13. Yotov, W.V. and St-Arnaud, R. (1992) Nucleotide sequence of a mouse cDNA encoding the nonhistone chromosomal high mobility group protein-1 (HMG1). *Nucleic Acids Research* **20**, 3516.
14. Ferrari, S., Finelli, P., Rocchi, M. and Bianchi, M.E. (1996) The active gene that encodes human high mobility group 1 protein (HMG1) contains introns and maps to chromosome 13. *Genomics* **35**, 367–371.
15. Mosevitsky, M.I., Novitskaya, V.A., Iogannsen, M.G. *et al.* (1989) Tissue specificity of nucleo-cytoplasmic distribution of HMG1 and HMG2 proteins and their probable functions. *European Journal of Biochemistry* **185**, 303–310.
16. Schröter, H. and Bode, J. (1982) The binding sites for large and small high-mobility-group (HMG) proteins. *European Journal of Biochemistry* **127**, 429–436.
17. Paull, T.T., Haykinson, M.J. and Johnson, R.C. (1993) The non-specific DNA-binding and -bending proteins HMG1 and HMG2 promote the assembly of complex nucleoprotein structures. *Genes and Development* **7**, 1521–1534.
18. Bustin, M. (1999) Regulation of DNA-dependent activities by the functional motifs of the high-mobility-group chromosomal proteins. *Molecular and Cellular Biology* **19**, 5237–5246.
19. Travers, A. (2003) Priming the nucleosome: a role for HMGB proteins? *EMBO Reports* **4**, 131–136.
20. Assenberg, R., Webb, M., Connolly, E. *et al.* (2008) A critical role in structure-specific DNA binding for the acetylatable lysine residues in HMGB1. *Biochemical Journal* **411**, 553–561.
21. Bonaldi, T., Längst, G., Strohner, R. *et al.* (2002) The DNA chaperone HMGB1 facilitates ACF/CHRAC-dependent nucleosome sliding. *EMBO Journal* **21**, 6865–6873.
22. Zappavigna, V., Galciola, L., Helmer Citterich, M. *et al.* (1996) HMG1 coopoerates with HOX proteins in DNA binding and transcriptional activation. *EMBO Journal* **15**, 4981–4991.
23. Agarwal, A. and Schatz, D.G. (1997) RAG1 and RAG2 form a stable postcleavage synaptic complex with DNA containing signal ends in V(D)J recombination. *Cell* **89**, 43–53.
24. Jayarman, L., Moorthy, N.C., Murthy, K.G. *et al.* (1998) High mobility group protein-1 (HMG-1) is a unique activator of p53. *Genes and Development* **12**, 462–472.
25. He, Q., Liang, C.H. and Lippard, S.J. (2000) Steroid hormones induce HMG1 overexpression and sensitize breast cancer cells to cisplatin and carboplatin. *Proceedings of the National Academy of Sciences, USA* **97**, 5768–5772.
26. Calogero, S., Grassi, F., Aguzzi, A. *et al.* (1999) The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycemia in newborn mice. *Nature Genetics* **22**, 276–280.
27. Falciola, L., Spada, F., Calogero, S. *et al.* (1997) High mobility group 1 protein is not stably associated with the chromosomes of somatic cells. *Journal of Cell Biology* **137**, 19–26.
28. Scaffidi, P., Mistell, T. and Bianchi, M. (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* **418**, 191–195.
29. Wang, H., Bloom, O., Zhang, M. *et al.* (1999) HMG-1 as a late mediator of endotoxin lethality in mice. *Science* **285**, 248–251.

30. Wang, H., Yang, H. and Tracey, K.J. (2004) Extracellular role of HMGB1 in inflammation and sepsis. *Journal of Internal Medicine* **255**, 320–331.
31. Bianchi, M.E. (2007) DAMPs, PAMPs and alarmins: all we need to know about danger. *Journal of Leukocyte Biology* **81**, 1–5.
32. Hori, O., Brett, J., Slattery, T. *et al.* (1995) The receptor for advanced glycation end products (RAGE) is a cellular binding site for Amphoterin. *Journal of Biological Chemistry* **270**, 25752–25761.
33. Kokkola, R., Andersson, A., Mullins, G. *et al.* (2005) RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages. *Scandinavian Journal of Immunology* **61**, 1–9.
34. Gardella, S., Andrei, C., Ferrera, D. *et al.* (2002) The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. *EMBO Reports* **3**, 995–1001.
35. Bonaldi, T., Talamo, F., Scaffidi, P. *et al.* (2003) Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. *EMBO Journal* **22**, 5551–5560.
36. Jiang, W., Li, J., Gallowitsch-Puerta, M., Tracey, K.J. *et al.* (2005) The effects of CpG DNA on HMGB1 release by murine macrophage cell lines. *Journal of Leukocyte Biology* **78**, 930–936.
37. Youn, J.H. and Shin, J.-S. (2006) Nucleocytoplasmic shuttling of HMGB1 is regulated by phospohorylation that redirects it to secretion. *Journal of Immunology* **177**, 7889–7897.
38. Ito, I., Fukazawa, J. and Yoshida, M. (2007) Post-translational methylation of high mobility group box 1 (HMGB1) causes its cytoplasmic localization in neutrophils. *Journal of Biological Chemistry* **22**, 16336–16344.
39. Hoppe, G., Talcott, K.E., Bhattacharya, S.K. *et al.* (2006) Molecular basis for the redox control of nuclear transport of the structural chromatin protein HMGB1. *Experimental Cell Research* **312**, 3526–3538.
40. Rovere-Querini, P., Capobianco, A., Scaffidi, P. *et al.* (2004) HMGB1 is an endogenous immune adjuvant released by necrotic cells. *EMBO Reports* **5**, 825–830.
41. Ditsworth, D., Zong, W.-X. and Thompson, C.B. (2007) Activation of poly(ADP)-ribose polymerase (PARP-1) induces release of the pro-inflammatory mediator HMGB1 from the nucleus. *Journal of Biological Chemistry* **282**, 17845–17854.
42. Bell, C.W., Jiang, W., Reich, C.F. *et al.* (2006) The extracellular release of HMGB1 during apoptotic cell death. *American Journal of Physiology Cell Physiology* **291**, C1318–C1325.
43. Popovic, K., Ek, M., Espinosa, A. *et al.* (2005) Increased expression of the novel proinflammatory cytokine high mobility group box chromosomal protein 1 in skin lesions of patients with lupus erythematosus. *Arthritis and Rheumatism* **52**, 3639–3645.
44. Qin, S., Wang, H., Yuan, R. *et al.* (2006) Role of HMGB1 in apoptosis-mediated sepsis lethality. *Journal of Experimental Medicine* **203**, 1637–1642.
45. Lee, E., Nakatsuma, A., Hiraoka, R. *et al.* (1999) Involvement of histone phosphorylation in thyomocyte apoptosis by protein phosphatase inhibitors. *IUBMB Life* **48**, 79–83.
46. Jiang, W., Bell, C.W. and Pisetsky, D.S. (2007) The relationship between apoptosis and high-mobility group protein 1 release from murine macrophages stimulated with lipopolysaccharide or polyinosinic-polycytidylic acid. *Journal of Immunology* **178**, 6495–6503.
47. Kokkola, R., Sundberg, E., Ulfgren, A.K. *et al.* (2002) High mobility group box chromosomal protein 1: a novel proinflammatory mediator in synovitis. *Arthritis & Rheumatism* **46**, 2598–2603.
48. Andersson, U. and Erlandsson-Harris, H. (2004) HMGB1 is a potent trigger of arthritis. *Journal of Internal Medicine* **255**, 344–350.
49. Ek, M., Popovic, K., Erlandsson-Harris, H. *et al.* (2006) Increased extracellular levels of the novel proinflammatory cytokine high mobility group box chromosomal protein 1 in minor salivary glands of patients with Sjögren's Syndrome. *Arthritis & Rheumatism* **54**, 2289–2294.

50. Goldstein, R.S., Gallowitsch-Puerta, M., Yang, L. *et al.* (2006) Elevated high-mobility group box 1 levels in patients with cerebral and myocardial ischemia. *Shock* **25**, 571–574.
51. Kim, J.-B., Choi, J.S., Yu, Y.-M. *et al.* (2006) HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. *Journal of Neuroscience* **26**, 6413–6421.
52. Jiang, W. and Pisetsky, D.S. (2007) Mechanisms of Disease: the role of high-mobility group protein 1 in the pathogenesis of inflammatory arthritis. *Nature Clinical Practice Rheumatology* **3**, 52–58.
53. Båve, U., Magnusson, M., Eloranta, M.-L. *et al.* (2003) FcγRIIa is expressed on natural IFN-α-producing cells (plasmacytoid dendritic cells) and is required for the IFN-α production induced by apoptotic cells combined with Lupus IgGs. *Journal of Immunology* **171**, 3296–3302.
54. Means, T.K., Latz, E., Hayashi, F. *et al.* (2005) Human lupus antibody – DNA complexes activate DCs through cooperation of CD32 and TLR9. *Journal of Clinical Investigation* **115**, 407–417.
55. Tian, J., Avalos, A.M., Mao, S.Y. *et al.* (2007) Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. *Nature Immunology* **8**, 487–496.
56. Czura, C.J., Yang, H. and Tracey, J. (2003) High mobility group box-1 as a therapeutic target downstream of tumor necrosis factor. *Journal of Infectious Diseases* **187**, S391–S396.
57. Andersson, U. and Tracey, K.J. (2004) HMGB1 as a mediator of necrosis-induced inflammation and a therapeutic target in arthritis. *Rheumatic Disease Clinics of North America* **30**, 627–637.
58. Sama, A.E., D'Amore, J., Ward, M.F. *et al.* (2004) Bench to Bedside: HMGB1 – a novel proinflammatory cytokine and potential therapeutic target for septic patients in the emergency department. *Academic Emergency Medicine* **11**, 867–873.
59. Yang, H., Ochani, M., Li, J. *et al.* (2004) Reversing established sepsis with antagonists of endogenous HMGB1. *Proceedings of the National Academy of Sciences, USA* **101**, 296–301.
60. Park, J.S., Svetkauskaite, D., He, H. *et al.* (2004) Involvement of TLR2 and TLR4 in cellular activation by high mobility group box 1 protein (HMGB1). *Journal of Biological Chemistry* **279**, 7370–7376.
61. Kokkola, R., Li, J., Sundberg, E. *et al.* (2003) Successful treatment of collagen-induced arthritis in mice and rats by targeting extracellular high mobility group box chromosomal protein 1 activity. *Arthritis & Rheumatism* **48**, 2052–2058.
62. Zetterström, C.K., Jiang, W., Wähämaa, H. *et al.* (2008) Pivotal advance: inhibition of HMGB1 nuclear translocation as a mechanism for the anti-rheumatic effects of gold sodium thiomalate. *Journal of Leukocyte Biology* **83**, 31–38.
63. Ostberg, T., Wähämaa, H., Palmblad, K. *et al.* (2008) Oxaliplatin retains HMGB1 intranuclearly and ameliorates collagen type II-induced arthritis. *Arthritis Research & Therapy* **10**, R1, doi:10.1186/ar2347.

Abstract

High-mobility group box 1 (HMGB1) functions as an alarmin following release from activated or necrotic cells and mediates inflammation. Translocation and extracellular release of HMGB1 can also occur during apoptosis. This translocation of HMGB1 from the nucleus to the cytoplasm results from post-translational modifications similar to those affecting histones. Such post-translational modifications of HMGB1 could also have effects on gene expression following changes in its DNA-binding properties. Furthermore, modified HMGB1 in the extracellular environment displays immunological activity and could serve as a potential target for new therapy.

Key words: pro-inflammatory; cytokine; amphoterin; chromatin; autoimmune; sepsis.

11

Idiosyncratic drug-induced liver injury: facts and perspectives

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11.1 Introduction

Compounds capable of eliciting liver injury are generally known as *hepatotoxins*. A significant number of compounds, among them industrial chemicals, drugs and herbal extracts, have been reported to cause damage to the liver that varies in intensity and severity. Although most frequently referred to parenchymal cells (hepatocytes), the injury elicited by hepatotoxins can also affect other liver cells (i.e. endothelial, ductal cells, etc.), resulting in different clinical forms of acute and chronic liver affectation. Drug-induced liver injury shares clinical features indistinguishable from other types of liver disease, such as acute and chronic hepatitis, alcoholic liver disease and cholestasic liver disease that occasionally can auto-perpetuate in the absence of the drug as an autoimmune disease. Drug-induced liver injury is the most common reason cited for withdrawal of an approved drug [1–3].

11.2 Intrinsic drug toxicity to the liver

The ways in which drugs damage the liver are certainly complex, and often poorly understood. Some of these compounds (*intrinsic* hepatotoxins, type A hepatotoxins)

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can cause effects in almost any individual beyond a certain dose, their effects beginning to be noticeable within a hours of exposure. A dose–response relationship is generally found and the toxic effects can be reproduced to a large extent in experimental animals. Consequently, these compounds behave in a rather predictable manner in humans.

This type of hepatotoxicity is most frequently found in occupational, environmental or household chemicals. Only a few drugs under clinical use are intrinsically and significantly toxic, such as chemotherapeutic agents and acetaminophen. Their mode of action is generally the consequence of a specific action of the molecule on the hepatocyte metabolism causing alterations of the cell substructures and biomolecules and/or biochemical malfunction. Interestingly, although the liver is remarkable for its ability to reduce the toxicity of xenobiotics compounds, the toxicity of which occurs as a consequence of (or in the course of) their biotransformation reactions catalysed by a group of drug-metabolizing enzymes – namely, cytochome P450 (CYP) monooxygenases and flavin monooxygenases, UDP glucuronil transferases (UDPGTs), glutathione-S-transferase and N-acetyl transferase –may result in the production of more toxic and/or reactive intermediates. Such a process is known as *bioactivation* [3].

Clinically, a cytotoxic pattern, a cholestasic pattern, or both, can occur [1,4]. Acute hepatocellular injury is the most common hallmark, but damage to other cell types (cholangiocytes, endothelial cells) is also possible. Drug-induced hepatic damage can be assessed by an increase (more than two times) of the upper normal limit of serum alanine aminotransferase (ALT/GPT), aspartate aminotransferase (AST/GOT), alkaline phosphatase (AP) and γ-glutamyl transpeptidase. In acute hepatocellular injury ALT elevation tends to be predominant, whereas in the cholestasic pattern bilirubin, AP and γ-glutamyl transpeptidase increase. The appearance of jaundice (total bilirubin level elevated by more than two times and ALT more than three times, or ALT/AP ratio more than five times) is a warning clinical sign for immediate discontinuation of drug administration [2,3,5–8].

11.3 Idiosyncratic drug toxicity to the liver

Other hepatotoxins, known as *idiosyncratic* hepatotoxins (or type B hepatotoxins), cause damage only rarely and in susceptible individuals. Idiosyncratic reactions are hardly predictable and are caused by the inability of certain individuals to tolerate the compound. Generally, the injury becomes noticeable within days or weeks after administration of the compound (occasionally it may be delayed for several months after drug exposure) and persists for months.

Several features characterize idiosyncratic reactions: they are closely linked to the structure of the responsible compound, rather than to its pharmacological properties; the clinical manifestations are highly dependent on the exposed individual and have a much lower occurrence than those of intrinsic hepatotoxins; the effects of the drug tend to be exaggerated and not proportional to the administered doses, which were well

tolerated prior to the sensitization process; although this type reactions can also occur in animals, the idiosyncratic nature of a drug for men cannot be reliably revealed in laboratory animals [1,4,9–11].

Zimmerman first classified idiosyncratic liver toxicity as metabolic and immunological idiosyncrasy and this classification is still widely acknowledged [2]. The former type is related to individual differences in the metabolism of a drug, which may result in the formation/accumulation of an unusual, more harmful, metabolite. The designation of metabolic idiosyncrasy is based on a lack of rash, fever and/or eosinophilia associated with toxicity as well to a lack of immediate toxicity on rechallenge [1,4,12]. This type of idiosyncratic reaction is attributable to pharmacogenetic differences between individuals resulting in differences in the metabolism of compounds and formation/accumulation of toxic metabolites [13]. This form of adverse drug reaction frequently develops within one to several weeks after initiation of drug therapy and is not accompanied by the characteristic features of an allergic reaction. Since the adverse effect is presumed to be the result of the formation and/or accumulation of toxic metabolites, rechallenge to elicit the hepatic injury usually requires administration of the drug for days to weeks [4]. It is estimated that 13–17% of acute liver failures can be attributed to idiosyncratic drug toxicity [4].

Otherwise, some adverse idiosyncratic hepatic drug reactions have clinical hallmarks of an immunological hypersensitivity mechanism [14], which includes delay in the time of presentation after the initial exposure, but rapid and exaggerated responses of sensitized individuals to previously well-tolerated doses. This is accompanied by laboratory evidence of immunological alterations, either in a given organ or as a part of a generalized hypersensitivity syndrome.

Drug-induced allergic hepatitis is a liver-specific inflammatory reaction elicited by a given drug and which is accompanied by typical features of a hypersensitivity reaction [4,15,16]. Allergic hepatitis is frequently associated with fever, rash and liver-cell infiltration (called DRESS syndrome) [17]. Although less common than other forms of drug-induced hepatotoxicity, it has more serious clinical implications and the outcome can sometimes be fatal [10,18].

By definition, a true hypersensitivity response to a drug requires the development of an immune response directed towards the molecule. The onset of a hypersensitivity reaction frequently involves the generation of drug-derived antigens resulting from covalent binding of the drug or reactive metabolites to proteins to form immunogenic conjugates, followed by antigen uptake, processing, presentation and T cell proliferation [1,19]. Although the immune response is similar to that elicited against any antigen, there are aspects that make the interaction of drugs with the immune system unusual [20]. First, drug-induced allergic hepatitis [16,21,22] shares many of the mechanistic features observed in other type IV reactions [23], involving $CD4^+$ $CD8^+$ cytotoxic T lymphocytes as well natural killer (NK) cells. Indeed, mild fever, eosinophilia and atypical lymphocytosis and liver infiltrate are frequently observed in those patients, together with the presence of sensitized T lymphocytes. Second, an antibody-type response can also occur (type-II hypersensitivity), but to a lesser extent. This is inferred, in part, from the presence of circulating antibodies in a patient's sera, either

directed against the drug [24] or against hepatic antigens [25–27], which increase promptly upon re-challenge with the suspected drug. Antibodies directed to the drug are much less common and the role of these antibodies in the pathogenesis of the liver disease is unclear [28]. Antibodies against cellular components occur when the sensitization process evolves towards an autoimmune reaction [29,30].

Characteristic drugs causing allergic-type reactions in the liver include a wide variety of compounds (antibiotics, sulphonamides, halogenated anaesthetics, non-steroidal anti-inflammatory drugs (NSAIDs), anticonvulsants and anti-depressive drugs), and they are capable of triggering hepatocellular damage, cholestasis or mixed reactions [27]. Other recently described idiosyncratic hepatotoxic drugs are presented in Table 11.1. Recovery after drug withdrawal may be delayed up to 1 year, in particular in cholestatic liver injury (in some cases associated with a vanishing bile duct syndrome). Most episodes have good clinical prognosis upon drug discontinuation. In certain cases the damage to liver cells may continue even upon drug withdrawal, and perpetuates in the form of autoimmune hepatitis (Table 11.1). Overall, chronic disease may occur in up to 6% of cases, even if the offending drug is withdrawn [4].

11.4 Mechanisms of hypersensitivity reactions to drugs in the liver

There are several working hypotheses to explain the onset of drug-induced allergic phenomena. The *hapten hypothesis* [29] postulates that drugs (or reactive moieties derived from the drugs) can react with cell proteins forming covalent adducts, a phenomenon known as *haptenization* [31] (Figure 11.1). The formation of drug-derived reactive intermediates in hepatocytes, a process known as *bioactivation,* can occur either in the course of biotransformation reactions catalysed by phase I drug-metabolizing enzymes, such as CYP monooxygenases or flavin monooxygenase enzymes, or by the formation of reactive conjugates with glutathione (GSH) or glucuronic acid in phase II reactions, resulting in the formation of stable immunoreactive drug adducts [32] (Figure 11.2). The extent of drug covalent binding is dependent on the proportion of the chemical that is converted into a reactive metabolite, its half-life, the reactivity towards available functional groups of biomolecules and the ability of the cells to sequester (neutralize?) these intermediates with endogenous molecules, such as GSH [31,33–37]. This is a general mechanism that apparently does not differ too much within tissues with respect to the formation of drug–protein adducts [38] that can trigger immune-mediated idiosyncratic hepatotoxicity [12,39–41].

Following this step, it is required that these neoantigens become accessible to the surveillance of the immune system. The *classic* pathway assumes that those adducts are captured, internalized, processed and presented by professional antigen-presenting cells (APCs) to $CD4^+$ and $CD8^+$ cells [31] (Figure 11.1).

In the liver, the formation of drug–protein adducts is not an unusual phenomenon, and the fact that anti-drug antibodies can be identified in the course of an hepatic allergy indicates that indeed a drug can act as a hapten. Yet, not all drugs that covalently

Table 11.1 Compounds recently reported to have caused allergic and/or drug-induced autoimmune hepatitis (2002–2007). Characteristic drugs causing immune-mediated reactions in the liver include a wide variety of compounds such as sulphonamides, halogenated anaesthetics, tienilic acid, and dihydralazine. For review, see [27,94,95].

Drug (prop\erty)	Clinical features	Laboratory data	Biopsy	Others	References
Allopurinol (anti-hyperuricemic)	Exfoliative dermatitis, fever, hepatitis and interstitial nephritis	Eosinophilia, increased AST, ALT, LDH, AP and BR	Not performed	Sustained improvement after prolonged therapy with steroids	[17,49,127]
Carbamazepine (anticonvulsivant)	Morbiliform macular rash, fever, induced fulminant liver failure	Increased CRP, AST, ALT, GGT, AP and BR	Not performed	Progress towards fulminant hepatic syndrome	[49,128]
Cetirizine (anti-H1 receptor antagonist)	Weakness, nausea, anorexia and hyperchromic urine	Increased ALT, AST, AP and total-BR; positive liver-kidney microsome antibodies	Diffuse portal tract and lobular inflammation with a prominent eosinophilic infiltrate	Onset after 6 days of therapy with oral cetirizine	[108]
Dapsone (leprosy drug)	Jaundice, fever, Stevens–Johnson syndrome-like	Increased of serum liver enzymes and BR, eosinophilia	Inflammatory cell infiltrate in cholangiolar epithelium	Onset after 3 weeks of therapy	[129,130]
Fluindione (vitamin K antagonist, oral anticoagulant)	Clinical features of acute liver failure	Positive skin-patch test	Mixed-type hepatitis with cytolysis and cholestasis	On reintroduction, rapid recurrence of clinical biological signs with increased severity	[131]
Gold salts (antiarthritic)	Acute cholestasic hepatitis, hypersensitivity features, dermatitis	Eosinophilia	Marked inflammation, ductopenia	Three weeks after onset of treatment, prolonged cholestasis still present after 15 months, probable immunoallergic mechanism	[132]

(*continued*)

Table 11.1 (*Continued*)

Drug (prop\erty)	Clinical features	Laboratory data	Biopsy	Others	References
Halogenated volatile anaesthetic drugs	Idiosyncratic drug-induced hepatitis	Autoantibodies (IgGs, IgG4), decreased complement C3a and C5a	Not performed	Small non-precipitating immune complexes that escape clearance	[26,99]
Indometacin (NSAID)	Toxic hepatitis evolving to autoimmune hepatitis	30–40-times increase of ALT, ASP; 10-times increase AP; 30-times increase BR; negative results for antibodies against rheumatoid factor, ASMAs, LKMAs	Lobular disarray, periportal and centrilobular hepatocyte dropout; subsequent biopsy showed a dense mononuclear cell infiltrate in portal tract	Great improvement with prednisone treatment followed by azathioprine and micophenolate	[97]
Infliximab (anti-TNF-α therapy)	Severe hepatitis with features of autoimmune disease	Increased ALT and AST; Increased ESR and CRP; elevated ASMAa, anti-dsDNA antibodies and ANAs	Intense, diffuse portal lymphoplasmacytic, granulocytic infiltration; severe interface hepatitis	Sudden appearance after the sixth infusion; history of previous psoriatic arthritis	[102,107]
Lamotrigine (anticonvulsivant)	Headache, vomiting, diarrhoea, fever, maculopapular rash, jaundice	Elevated ALT, AST; leucocytosis; negative serum test for HAV, HBV, CMV and EBV	Mixed portal infiltrate (lymphocytes, neutrophils, eosinophils); diffuse interface hepatitis	Late onset (after 20 days' treatment); previous episode of hypersensitivity to carbamazepine	[133]
Metamizole (analgesic)	Generalized exanthema and influenza-like symptoms	Elevated AST, ALT and AP	Perivenular non-bridging confluent necrosis and granuloma formation; acute hepatitis	Positive LTT to metamizole and metabolites	[134]

Minocycline	Severe jaundice and general malaise	Increase in serum liver enzymes, eosinophilia and ANAs; elevated IgGs	Inflammatory cells infiltrate in portal tract	Onset 12 months after initiating treatment	[96,100]
Nevirapine (antiretroviral agent)	Fever, toxic epidermal necrolysis, drug rash with eosinophilia and systemic symptoms acute liver failure	Eosinophilia, elevated serum liver enzymes	Not performed	3–6 weeks after treatment, hepatic toxicity in HCV-co-infected males	[135–138]
Rifampin (tuberculostatic)	Autoimmune hepatitis and thyroiditis	Very increased ALT and AST, normal levels of BR, elevated ANA, ASMA antithyroid and antithyroglobulin antibodies, eosinophillia	Not performed	Associated with the use of pyrazinamide	[94]
Statins (anti-cholesterolemic)	Liver injury with characteristics of an autoimmune disease	Elevated serum liver enzymes; ANA, ASMA,hypergamma-globulinaemia	Varying stages of fibrosis and plasma cell infiltration	Weeks to months after treatment	[3,73,98, 103,106,109]
Telithromycin (antibiotic)	Acute cholestasis with mild parenchymal damage	Two-times increase in ALT, AST; AP, GGT normal, increased BR (20 times)	Not performed	Rapid onset, a previous episode of acute hepatitis after drug use	[139]
Twinrix® (inactivated HVA + rHBsAg vaccine)	Severe jaundice	Elevated serum liver enzymes, BR; increased IgGs and ANA	Marked bridging fibrosis, moderate chronic infiltrate	Exposure to vaccine led to exacerbation of chronic liver disease	[101]

ANAs, anti-nuclear antibodies; anti-dsDNA, anti-double-stranded DNA; ASMAs, anti-smooth muscle antibodies; BR, (total) bilirubin; CMV, cytomegalovirus; CRP, C-reactive protein (acute phase protein); EBV, Epstein–Barr virus; ESR, erythrocyte sedimentation rate; GGT, γ-glutamyl transpeptidase; HCV, hepatitis C virus; HAV, hepatitis A virus; HBV, hepatitis B virus; LDH, lactate dehydrogenase; LKMAs, liver-kidney microsomal autoantibodies; LTT, lymphocyte transformation test; NSAID, non-steroidal anti-inflammatory drug.

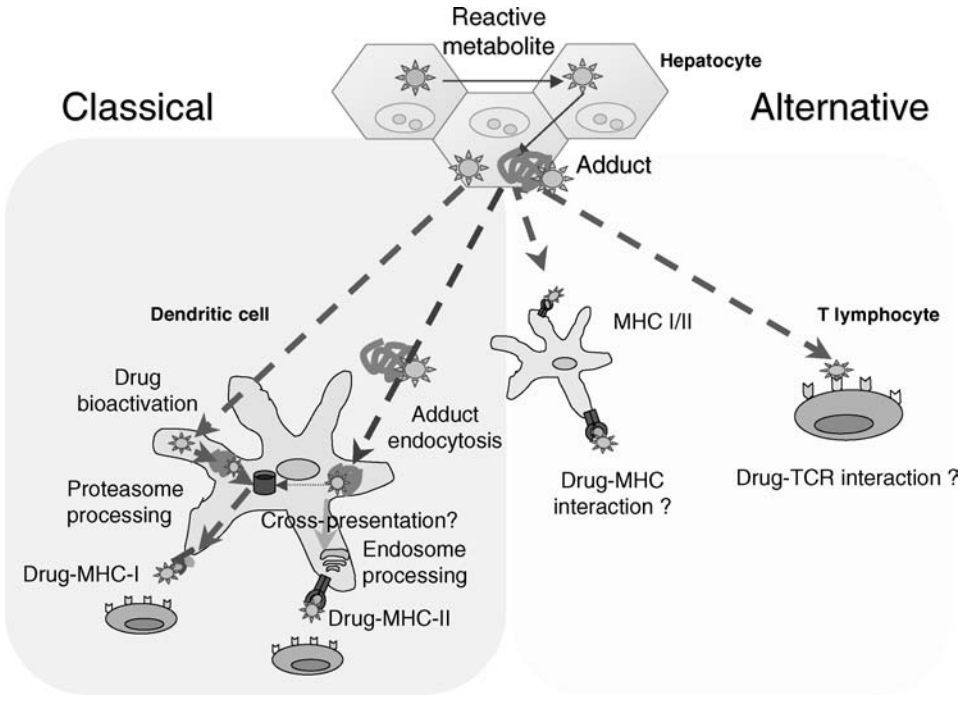

Figure 11.1 Mechanisms of sensitization and immune response elicitation in drug-induced allergic hepatitis. Bioactivation of drugs, formation of drug–protein adducts by hepatocytes, antigen uptake and presentation to T helper (Th) cells in the context of MHC molecules by APCs is a possible pathway that can initiate the immunological response. Alternatively, direct interaction of the drug with MHC molecules and/or TCR can also operate in certain cases.

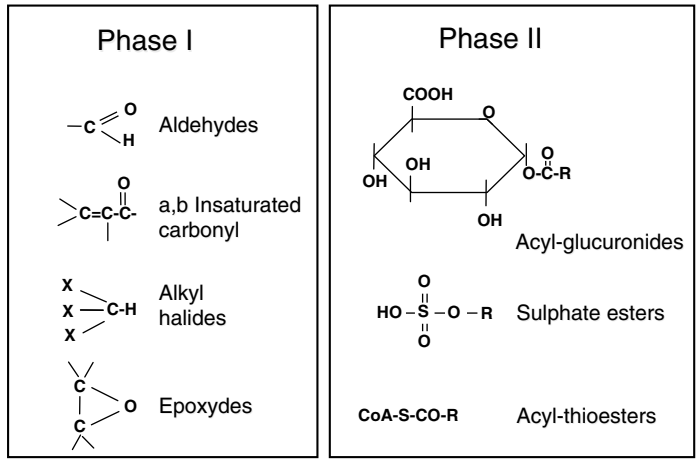

Figure 11.2 Reactive intermediates frequently involved in the formation of drug–protein adducts. Both phase I- and phase II metabolites can participate in the formation of drug–protein adducts. Phase I reactive metabolites tend to be electrophiles that react with nucleophilic moieties of the protein ($-NH_2$, $-SH$). Phase II reactive metabolites are most frequently unstable esters which undergo nucleophilic displacement by $-NH_2$ groups.

bind to hepatic proteins to a certain extent are associated with a significant incidence of hepatotoxicity in humans and many drugs that significantly form protein adducts cause idiosyncratic drug reactions only rarely. Current knowledge indicates that metabolic activation of a drug to a reactive intermediate and adduct formation is required, yet insufficient, in the onset of an idiosyncratic reaction [31,33–38,42–44]. Consequently, the formation of adducts is not the *unique* determinant of the whole process and additional signals seem to be required to break the immune tolerance to the liver.

To explain the need for a second signal, the *danger hypothesis* was proposed by Matzinger [45]. This hypothesis foresees that, in addition to recognition of the foreignness of the drug adduct, concomitant alarm signals (i.e. cell damage caused by the drug or its metabolites) are needed to upregulate the expression of co-stimulatory factors required to induce a subsequent immune response [46–48]. Indeed, it is remarkable that drugs (i.e. halothane) that are associated with idiosyncratic reactions frequently cause mild reversible liver injury in exposed patients and in many cases the allergic reaction may be preceded by a non-immune hepatic injury. The major controversy around this view is, however, the fact that the vast majority of patients do not show immune-based drug idiosyncratic reactions even in a context prone to create danger signals; that is, surgery or inflammation.

Some molecules are reported to interact and stimulate immune cells without the evidence of prior drug–adduct formation. The *pharmacological interaction hypothesis*, first formulated by Pichler (reviewed in [48]), aims to explain the existence of reactive T clones in patients with a history of drug hypersensitivity reaction, capable of proliferating in the presence of the drug, but apparently not requiring prior formation of drug covalent adducts [49]. According to this hypothesis, it is conceivable that certain compounds (reversibly?) interact with the major histocompatibility complex (MHC)–T cell receptor (TCR) complex, and induce an immune response [48] (Figure 11.3).

Current views on the phenomenon idiosyncratic drug reactions tend to integrate the various hypotheses as being complementary, rather than exclusive of each other. It is very likely that the onset of a hypersensitivity reaction may involve the co-participation of these mechanisms at different stages [1,19]. In this way, formation and presentation of drug–protein adducts, or direct interaction of the drug with the MHC–TCR complex would be a *necessary*, but not *sufficient* stimulus to trigger the hypersensitivity reaction. Several immunoallergic responses are initiated or intensified under concomitant inflammatory states or unrelated immune stimuli [1,48]. Cell injury caused by the drug itself, a concomitant inflammatory process, or a coincidental stimulus – that is, a viral infection – may represent the *additional* signal needed to initiate the immunoallergic process.

11.5 Hypersensitivity versus tolerance

The incidence of immune-mediated drug hepatotoxicity is relatively low, and this is probably a consequence of the fact that the cell microenvironment of the liver is believed to favour immune tolerance rather than inflammatory immunity [50].

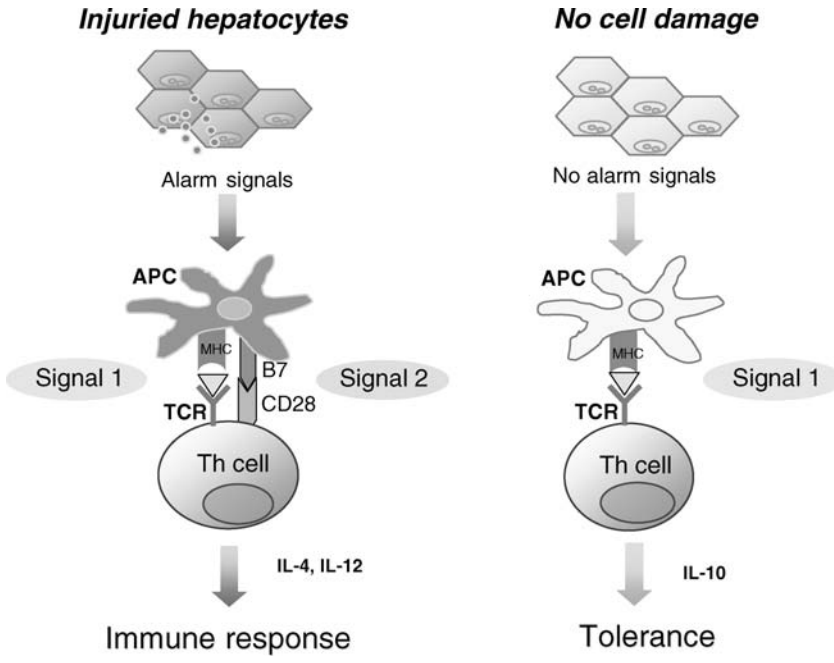

Figure 11.3 Immune response versus tolerance. The liver shows considerable tolerance to foreign antigens. The mechanisms of induction and maintenance of tolerance in self-reactive T cells in the periphery are poorly understood. Current knowledge assumes that successful T cell activation by APCs occurs only if, in addition to TCR recognition of the antigen (signal 1), a co-stimulatory signal also operates (signal 2) as a consequence of hepatocyte injury. In the absence of signal 2, signal 1 is either ignored or induces tolerance. Evidence exists indicating that hepatic dendritic cells, Kupffer cells, sinusoidal endothelial cells and possibly hepatocytes can carry out antigen presentation, but at the same time they can also induce tolerance. (IL, interleukin)

Hepatocytes, because of their great capability to metabolize drugs, usually form drug–protein adducts, for which the immune system normally shows tolerance. This tolerogenic property may be attributable to different factors: the liver's capacity to produce cytokines, i.e. interleukin (IL)-4, IL-6, IL-10, IL-13 and IL-15 [51], and inhibitory factors, i.e. prostaglandins [52]; the antigen presentation and activation of naïve lymphocytes in the hepatic sinusoid and the tolerogenic role of other liver cells [53–57].

Hepatic sinusoids contain four resident cell populations which are relevant for immune and inflammatory responses: endothelial cells, macrophages (Kupffer cells), liver-specific NK (pit cells) and fat-storing cells, also called lipocytes or Ito cells [58]. Recent observations suggest a fifth intrasinusoidal population of dendritic cells [59,60]. In the liver, clearance of antigen from the blood occurs mainly by sinusoidal endothelial cells through a very efficient receptor-mediated endocytosis, while non-specific phagocytosis is mediated primarily by Kupffer cells. The mechanisms of

induction and maintenance of tolerance in self-reactive T cells in the periphery are poorly understood. Current knowledge assumes that successful T cell activation by APCs occurs only if, in addition to TCR recognition of the antigen (signal 1), a co-stimulatory signal also operates (signal 2); signal 1 in the absence of signal 2 is either ignored or induces tolerance [31,61] (Figure 11.3). Evidence exists indicating that hepatic dendritic cells, Kupffer cells, sinusoidal endothelial cells and possibly hepatocytes can carry out antigen presentation [62], but at the same time they can also induce tolerance [53,61].

Lymphocytes are in the first line of immunoallergic responses in the liver. The lymphocyte population of the liver is enriched in NK cells and NK T cells (NKT cells), which appear to have crucial roles in the recruitment of circulating T cells [63]. Resident hepatic T cells are phenotypically different from T cells in the blood, lymph nodes or the spleen. In the liver the CD4/CD8 ratio is reversed, and there is a higher percentage of $CD3^+CD4^+CD8^+$ and $CD3^+CD4^-CD8^-$ populations. More than 15% of $CD3^+$ lymphocytes of the human liver express TCR-$\gamma\delta\psi$ rather than TCR-$\alpha\beta\Psi$ receptors, compared to the smaller TCR-$\gamma\delta\psi$ population in the peripheral blood. These observations suggest the likelihood of a control of function and/or differentiation of lymphocyte populations in the liver [61].

One hypothesis [54] postulates that the balance between immunity and tolerance in the liver is determined by the site of primary activation of $CD8^+$ T cells. Whereas naive $CD8^+$ T cells, if properly activated within the lymph nodes, are capable of mediating experimental hepatitis, lymphocyte activation in the liver is relatively ineffective and cells exhibit a defective cytotoxic function, a shortened half-life and an inability to mediate hepatocellular injury. A large macrophage population and the efficient trafficking of dendritic cells from the sinusoidal blood to the lymph promote antigen trapping and T cell priming, but the local presentation of antigen causes T cell inactivation, tolerance and apoptosis [64].

The mechanisms determining this dual pathway of activation are unclear. Thus, the outcome of intrahepatic $CD8^+$ T cell responses might be determined by whether the primary activation occurred within the tolerogenic environment of the liver, or whether immunity was induced by antigens in an immune-activating environment, as encountered within the lymph nodes [65]. Decrease in the number of available lymphocytes may be also one of the mechanisms preventing subsequent occurrences of allergic hepatitis induced by hepatic drug–protein adducts [66].

Sinusoidal endothelial cells can directly interact with passenger leucocytes and are unique in the sense that they constitutively express all molecules necessary for antigen presentation: CD54, CD80, CD86, MHC class I and class II and CD40, transforming growth factor-β (TGF-β) and adhesion molecules such as intercellular cell adhesion molecule (ICAM-1 (CD54), ICAM-2 (CD102)) and vascular cell adhesion molecule 1 (VCAM-1). They can also function as APCs for $CD4^+$ and $CD8^+$ T cells [50]. On top of that, these cells express IL-1 and the co-stimulatory molecule B7 [67,68]. Thus, these cells probably contribute to hepatic immune surveillance by activation of effector T cells [61].

On the other hand, liver sinusoidal endothelial cells are principally active in the uptake and cross-presentation of oral antigens from the portal venous blood, but

mostly engaged in the induction of $CD8^+$ T cell tolerance towards these antigens. Experiments *in vitro* reveal that naive T cells are activated by resident sinusoidal endothelial cells, but do not differentiate into effector T cells. These T cells show a cytokine profile and a functional phenotype compatible with the induction of tolerance [56,57,65]. Liver sinusoidal lining cells can take up antigen, process and present it to T cells but, probably due to the lack of input from helper T cells, finally undergo tolerance rather than immunity. This major function of sinusoidal endothelial cells is likely to prevent immunological reactions against the wide spectrum of potentially antigenic molecules acquired through the gastrointestinal tract [56].

Kupffer cells play a major role in the clearance of gut-derived antigens and pathogens entering the liver with portal venous blood and can act in the liver as APCs. There are clear examples showing that Kupffer cells can internalize and process neoantigens generated by hepatocytes [67,69,70]. Kupffer cells can express MHC class II molecules, ICAM-1, as well as low levels of CD80 and CD86. Kupffer cells cells can function as APC for allo $CD4^+$ T cells and Th1 clones [71]. However, data showing that Kupffer cells may induce activation of naive $CD4^+$ or $CD8^+$ T cells is lacking. The antigen-presentation properties of Kupffer cells are regulated by reactive oxygen species (ROS) and the expression of MHC class II and co-stimulatory molecules in Kupffer cells can be blocked by inhibiting ROS generation, suggesting that intracellular endogenous ROS generation during antigen processing provides essential secondary signalling for Kupffer cell antigen presentation [72]. Kupffer cells can be activated by endotoxins (bacterial lipopolysaccharide) and bacterial superantigens as well, releasing cytokines that possibly modulate the antigen-presenting properties of other cells in drug-induced hepatotoxicity [67,68,73].

Kupffer cells are more likely to have a role as primary inducers of immunological tolerance against delayed T cell responses [55,74]. Pre-treatment of mice with 2,4-dinitrophenylated protein adducts led to its accumulation in Kupffer cells and to a subsequent tolerance to 2,4-dinitrochlorobenzene sensitization [55]. Indeed, liver tolerance is impaired if Kupffer cells are depleted [53]. One possible mechanism by which Kupffer cells may induce T cell tolerance is that although they can act as APCs they express inadequate levels of co-stimulatory molecules and are thus only partially competent, thereby leading to T cell anergy rather than activation. Indeed the expression of various APC-related molecules (MHC II, B7-1, B7-2 and CD40) on Kupffer cells is low compared with dendritic cells [75]. These findings suggest that genetic and/or environmental factors that cause impairment of the tolerogenic functions of Kupffer cells may lead to an increased risk of developing immune adverse drug reactions in certain individuals [76].

Dendritic cells are professional APCs. Although they represent only a small percentage of the total number of cells, they play a major role in the regulation of liver immunity. The liver contains different subtypes of resident dendritic cells ($CD8\alpha^-B220^-$), lymphoid cells ($CD8\alpha^+B220^-$), plasmacytoid cells ($CD8\alpha^-B220^+$) and NK dendritic cells ($CD8\alpha^-$B220-$NK1.1^+$) that are poor stimulators of naïve T cells [60]. They mature as they migrate from the portal vein to the central vein and contribute to the initiation of T cell-mediated immune responses in the liver. Hepatic

sinusoids serve to select and concentrate circulating dendritic cells into the hepatic regional lymph nodes, the major migration pathway for dendritic cells from the blood. Mature dendritic cells in the central region of the hepatic acini traverse the space of Disse and enter the hepatic lymph system [61]. Their presence in the hepatic sinusoid allows them to present hepatocyte-borne antigens to lymphocytes.

During maturation, dendritic cells increase by several-fold their surface expression of MHC II molecules, located mostly on the plasma membrane of mature cells. Mature dendritic cells can also present foreign antigens associated to MHC I proteins [77]. Immature and mature dendritic cells present antigens to T cells under steady-state and inflammatory conditions [78]. An important issue is whether those dendritic cells that reside in the hepatic portal tracts can be involved in local antigen presentation, or whether they must first traffic to the draining lymph nodes [53]. From a traditional point of view, dendritic cells should pick up antigens from the liver and traffic to regional lymph nodes where resting, naïve lymphocytes are preferentially located. Once activated, antigen-specific lymphocytes would enter the bloodstream and home to the liver to exert their effector functions. Alternatively, antigens may be presented *in situ* by liver-resident bone marrow-derived dendritic cells and activate infiltrating naive lymphocytes. The first, but not the second, pathway seems to give rise to fully effector cytotoxic $CD8^+$ cells [54].

Recent studies point to an important role for dendritic cells in the induction of peripheral tolerance as well [60]. It has been proposed that the role of dendritic cells in the immunity/tolerance decision could be associated simply with dendritic cell maturation states [79]. Two main hypotheses have been put forward to explain such a dichotomy in the behaviour of dendritic cells. The first hypothesis argues that the role of dendritic cells in the immunity/tolerance decision could be associated with dendritic cell maturation states; that is, immature dendritic cells lacking co-stimulation may induce tolerance. However it has been correctly pointed out that immature dendritic cells do not process endocytosed antigens sufficiently to form MHC–peptide complexes on the cell surface. Therefore, self-specific T cells would not be able to recognize their ligand on immature dendritic cells. Moreover, it was also shown that a maturation signal is necessary to induce migration of immature dendritic cells from peripheral tissue to local lymph nodes [53,78].

A second hypothesis points at dendritic cells having different maturation programmes in the absence or presence of danger signals: activated, mature dendritic cells induce T cell immunity, and resting, non-activated, but fully differentiated, mature antigen-presenting dendritic cells can induce tolerance, leading to mature-tolerogenic and mature-immunogenic phenotypes, respectively [79–81]. Cells of the dendritic family are suited to perform two distinct functions at two discrete locations. In peripheral tissues, dendritic cells act as sentinels for 'dangerous' antigens. Then they migrate into lymphoid organs where they initiate activation of T lymphocytes which are specific for these antigens. During their migration, dendritic cells shift from an antigen-capturing mode to a T cell-sensitizing mode. In addition to switching on the immune response, subtypes of dendritic cells appear to influence the Th1/Th2 balance [82].

Hepatocytes express few MHC class I molecules, and almost no MHC class II antigens. However, under certain clinical circumstances, they may exhibit aberrant MHC II expression [83]. In this context, interferon (IFN)-γ has also been reported to induce MHC II antigen expression [84–86]. Extension of hepatocellular microvilli through intercellular junctions between sinusoidal endothelial cells can allow direct contact of hepatocytes with naïve $CD8^+$ T cells. It is therefore conceivable that under certain circumstances (overexpression of MHC II proteins) the hepatocyte could behave as a non-professional accessory APC, contributing to the onset of the immunological response towards drugs. However, it is not clear whether MHC II-expressing hepatocytes are functionally apt to present antigen to $CD4^+$ T lymphocytes in that hepatocytes have been reported to lack co-stimulatory molecules such as CD40 and B7.2.

Other experiments revealed that T cell activation by hepatocytes leads to premature T cell death or tolerance, rather than to activation. T cells activated by hepatocellular antigen presentation are phenotypically different from those activated in the spleen or lymph nodes. Apoptosis of hepatocyte-activated T cells is suspected to be an example of death by neglect resulting from the absence of an effective co-stimulatory signal. Crosslinking of CD28 on hepatocyte-stimulated T cells abrogated the early apoptosis of T cells, caused an increase in expression of Bcl_{xL} and IL-2 in hepatocyte-activated T cells and resulted in sustained T cell proliferation and cytotoxic activity [61]. Therefore, MHC class II-expressing hepatocytes, as found in clinical hepatitis, can serve as APCs and stimulate $CD4^+$ T cells; antigen presentation by hepatocytes may thus influence the outcome of clinical hepatitis but is unlikely to cause hepatitis [86].

In this context, other results suggest that $CD4^+$ T cells from patients suffering form allergic hepatitis can be induced to proliferate in response to the combination of the drug and a specific protein from the hepatic membrane [87]. In the case of autoimmune hepatitis, stimulation of T cell clones by hepatocytes in a MHC-restricted manner has also been observed: only hepatocytes expressing MHC II could stimulate $CD4^+$ T clones, whereas hepatocytes expressing MHC I stimulated $CD8^+$ T clones [84].

11.6 Hepatocyte injury as a consequence of allergic hepatitis

The immune response to foreign antigens in the liver is generally associated with a strong and sustained CD4 and CD8 T cell response. Immune-mediated killing of hepatocytes is mainly achieved by cytotoxic T cells [31] (Figure 11.4). Activated $CD8^+$ T cells are recruited to and/or trapped in the liver irrespective of their antigen-specificity. Only upon recognition of their cognate antigen, however, do these $CD8^+$ T cells undergo rapid proliferation. Proliferation presumably occurs in the liver, as increased numbers of antigen-specific T cells are not detectable in draining lymph nodes during the early days of the hepatic response. The lytic activity of cytotoxic T lymphocytes can occur through at least two pathways. In the perforin-/granzyme-

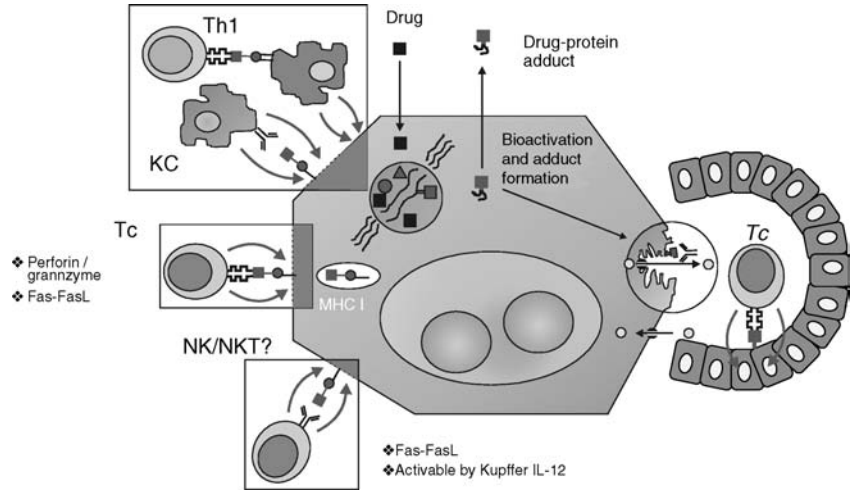

Figure 11.4 Mechanisms of liver damage in immune-mediated drug hepatotoxicity. The immune response to foreign antigens in the liver is generally associated with a strong and sustained T cell response. Immune-mediated killing of hepatocytes is mainly achieved by cytotoxic T cells and NK cells acting on the target cells. Drug-derived antigen formation as a result of metabolism and bioactivation reactions, when associated with MHC class I and transported to the cell's surface, result in a $CD8^+$ T cell (Tc) attack. Both parenchymal and ductal cells can be targets of the immune attack. NK cells, as well as Kupffer cells (KC), can also cause injury to hepatocytes. Their cytoplasm contains perforin and granzymes; and they can also act via Fas ligand-activating apoptosis. An antibody-type response to the drug can also occur, but to a lesser extent. Antibodies to cell substructures become noticeable when immune-mediated drug hepatotoxicity evolves towards autoimmune disease, and in some instances have been implicated in the onset of cholestasis.

mediated pathway, the pore-forming agent perforin, probably in conjunction with granzymes, induces apoptosis in target cells. In the Fas-mediated pathway, engagement of Fas and FasL triggers apoptosis of the cytotoxic T lymphocyte-bound target cell by a death domain-initiated caspase cascade [61].

NK cells and NKT cells are effector cells in the liver as well. NK cells are bone marrow-derived mononuclear cells that have markers of both T lymphocytes and macrophages. Their cytoplasmic granules contain perforin and granzymes, which are involved in cell membrane attack and induction of apoptosis in target cells. As opposed to target recognition by cytotoxic T lymphocytes, recognition of target cells by NK cells is not restricted to MHC-antigen presentation and their major role is the defence of the liver against invading tumour cells [61].

NKT cells are considered to be separate from NK and pit cell populations. In addition to exhibiting a NK phenotype, they express a T cell receptor that can interact with CD1, as opposed to the MHC I or MHC II interaction with the TCR on T lymphocytes, and can interact with target cells without restrictions. This liver-

resident, locally regenerating pool of rapid-response killing cells has a significant role in defending the liver from invading tumour cells [61].

Both NK and NKT cells are likely to play a role in the progression of drug-induced liver injury by secreting IFN-γ, and provoking a concomitant inflammatory response (chemokine production, accumulation of neutrophils and upregulation of FasL expression in the liver), thus contributing to the severity and progression of liver injury downstream of the metabolism of the hepatotoxic drug [88]. Nevertheless, NK and NKT cells have been reported to dramatically diminish in a case of fulminant drug hepatitis, suggesting that both NK and NKT cells might be involved in hepatic injury in fulminant hepatic failure [89].

The role of Kupffer cells in causing hepatocyte damage in the course of drug hepatotoxicity is ambivalent and likely to be indirect. These cells can be activated by different stimuli that result in the release of mediators acting on hepatocytes (tumour necrosis factor α (TNF-α), nitroc oxide, ROS) [26], with important catabolic effects on hepatocytes [90]. Activation of Kupffer cells seems to be one of the early events in acetaminophen toxicity, yet a protective effect on liver cells has also been described [76,91]. In general, damage to hepatocytes diminishes upon withdrawal of drug administration. However, certain episodes of idiosyncratic drug liver toxicity are remarkable in the sense that they can be perpetuated after drug discontinuation [92,93].

11.7 Drug-induced liver autoimmunity

Autoimmune hepatitis can be one of the most serious consequences of a drug-induced hepatitis, in which damage to the liver continues once the use of the drug has been discontinued. A number of drugs (i.e. diclofenac, methyldopa, nitrofurantoin, clometacin and interferon) were, early on, recognized as capable of causing autoimmune hepatitis [27]. In recent years more compounds have been identified (rifampin, minocycline, atorvastatin/ezetimibe and other statins) [27,94–109] (Table 11.1). The symptoms of drug-induced autoimmunity can resemble those of a typical systemic autoimmune diseases (i.e. systemic lupus erythematosus) [30,103,110], or be organ-specific (i.e. autoimmune hepatitis) [30].

There are two broad categories of immune-mediated liver disease: (1) autoimmune hepatitis with the features of hepatocellular disease and (2) those with features of a cholestasic liver disease. Drugs can induce both the hepatocellular and cholestasic forms of immunologically mediated liver disease and, if injury is not recognized promptly, they can give rise to chronic hepatitis (resembling viral hepatitis) or cholangitis (resembling primary biliary cirrhosis). Examples of the former type of drugs are α-methyldopa, halothane, hydralazine and other hydrazine-containing drugs, minocycline, nitrofurantoin, oxyphenizatin and statins [105].

Severe forms of drug-induced cholestasis may persist after the drug has been discontinued, and a small number of patients who develop drug-associated cholestasic hepatitis develop progressive self-destruction of cholangiocytes [111]. Cholangitis and/or chronic cholestasic syndromes with biochemical, serological and histological

features of primary biliary cirrhosis have also been described following exposure to certain drugs (chlorpromazine, dihydralazine, tienilic acid and halothane). Nevertheless, although many drugs can cause transient severe cholestasic hepatitis while being administered – that is, antibiotics, notably penicillins, cephalosporins, macrolides and antidepressants – they rarely, if ever, cause self-perpetuating autoimmune liver disease [105,111].

Autoimmune hepatitis elicited by drugs may clinically resemble type 1 or type 2 disease. Type 1 is characterized by high titres of anti-nuclear antibodies and anti-smooth muscle antibodies [112] and is the predominant form. Oxyphenizatin, nitrofurantoin, minocycline, α-methyldopa and clometacine as well as statins, among others, act in this manner (Table 11.1). Type 2 autoimmune hepatitis is characterized by an immune response directed towards drug-metabolizing enzymes (LKM-1/2/3, CYP2D6, CYP2C9, UGT1A and others) [112], and has been reported to be elicited by drugs such as dihydralazine, tienilic acid and halothane [92,93]. Although the auto-antibodies are used in classification, there is little evidence to support their role in the pathogenesis of the disease [112].

The differential diagnosis of the disease relies on several features. It is likely to be a drug-induced autoimmune hepatitis (1) when it is associated with high titres of anti-nuclear antibodies (>1/40), as well as other autoantibodies, and high serum immunoglobulin content that appeared after administration of the drug, (2) if the liver disease improves somewhat after drug withdrawal, but most frequently it will be self-sustaining with elevated hepatic enzyme markers that do not decrease without treatment, which is in contrast with drug hepatitis, (3) when specific histocompatibility locus antigen (HLA) genes not identified in the patient (i.e. HLA DRB1*0301 and DRB*0401), (4) when there is good responsiveness to corticoid/azathioprine treatment, (5) when there is recurrence of recovered liver disease upon drug re-challenge and (6) if other cases are reported in the literature for the drug under suspicion. On the contrary, the autoimmune hepatitis is more likely to be 'cryptogenic' if (1) the anti-nuclear antibody titre was elevated before drug administration, (2) specific HLA genes have been identified in the patient, (3) the affected person has another concomitant autoimmune disorder (i.e. Sjögren's syndrome, thyroiditis, arthritis, etc.) and (4) the suspected drug has not been reported in the literature (or there is only a single case report).

Assuming that the onset of liver injury resembling autoimmune hepatitis occurred temporally and is unequivocally associated with consumption of a given drug, still different questions can be raised. First is the question of whether there could be a pre-existing, but subclinical, autoimmune hepatitis that has now been identified for the first time, or whether it is the consequence of a drug that exacerbates known autoimmune hepatitis. Certainly, the drug may have induced *de novo* liver injury that manifests as an autoimmune hepatitis, but it is also possible for the drug to have behaved as the trigger for development of autoimmune hepatitis in a person with an inherited susceptibility. The availability of analytical data prior to disease manifestations (i.e. anti-nuclear antibodies, serum immunoglobulins) helps to discriminate among the different possibilities.

The causes driving the immune-mediated destruction of hepatic tissues in drug-induced autoimmune hepatitis are only partially known. The syndrome differs from typical drug hypersensitivity reactions in that drug-specific T cells or antibodies are not always present, and that episodes may even occur apparently without any immune sensitization to the drug [22,92,93,112]. A conceptual framework for the pathogenesis of autoimmune hepatitis points to environmental agents that could trigger a cascade of T cell-mediated events directed to liver antigens in a genetically predisposed host [93]. One possibility is that a reactive metabolite generated may covalently bind to a self-protein and form an hapten adduct capable of eliciting an adaptive immune response against the metabolite–self-protein complex that can break tolerance to self-proteins, thus leading to autoimmunity. According to this hypothesis, haptenization appears to be a first step but, since bioactivation occurs in many instances without immune triggering, drug-induced autoimmune hepatitis is likely to require an additional co-stimulatory event. For instance, the hepatocellular necrosis or apoptosis caused by toxic drug/metabolites can stimulate nearby cells (i.e. Kupffer cells) to secrete pro-inflammatory cytokines and to present autoantigens to adaptive T cells.

$CD4^+$ T lymphocytes expressing the IL-2 receptor α chain ($CD25^+$) appear to be central to the maintenance of self-tolerance by preventing the proliferation and effector functions of auto-reactive T cells [51]. In addition to $CD4^+$ T cells, there is growing evidence for a role for $CD8^+$ T cells [51]. It is also conceivable that, as in autoimmune hepatitis, regulatory T cells (Tregs, $CD4^+CD25^+$) are defective in number, but maintain the ability to suppress IFN-γ production. Tregs are unable to regulate $CD8^+$ T cell proliferation and cytokine production in patients studied at diagnosis, but they suppress CD8 T cell proliferation and induce an elevation of IL-4-producing $CD8^+$ T cells during remission [51]. The inability of Tregs to regulate $CD8^+$ T cell function may thus contribute to the initiation of autoimmune liver damage.

Most autoimmune diseases have an inherited component, and some of them cluster in families. The autoimmune regulator (AIRE), a gene located on chromosome 21q22.3 and associated with the development of the autoimmune polyglandular syndrome type 1 (APS-1), has also been related to autoimmune hepatitis disease, as 15–20% of APS-1 patients develop hepatitis. AIRE expression is highest in the medullar thymus epithelial cells and its structure suggests that it may act as a transcriptional regulator. Through the generation of AIRE-deficient mice that developed several autoimmune phenotypes it was possible to define its pathological role. AIRE upregulates the transcription of organ-specific self-antigens in medullary thymic epithelial cells to provide an antigen library against which central tolerance is developed [113]. So far, more than 50 different mutations of the AIRE gene have been identified. They are distributed throughout the entire non-coding and coding regions. AIRE was speculated to contribute to the onset of liver autoimmune disorders [114]. However it has not been possible to establish a clear link, and recent studies indicate that common mutations in the AIRE gene do not play a major role in autoimmune liver diseases [114].

Both types of drug-induced autoimmune hepatitis are treated satisfactorily with high doses of prednisone (1 mg/kg per day) that can be gradually lowered as the disease ameliorates. Treatment with prednisone (or in combination with azathio-

prine, 1–1.5 mg/kg/day) remains the standard therapy [115]. The goal is to reach the lowest possible dose that controls the disease, avoiding the side effects of the corticosteroid. Clinical and biochemical normalization usually occurs within 3–6 months, if the diagnosis is correct, but histological improvement may lag behind. The evolution of the disease is slow and it may take months to 1–3 years to remit. Drug treatment with low doses of prednison (or azathioprine) may be required for many months after the normalization of clinical parameters to keep the disease silent. In less severe cases, treatment may begin with both azathioprine and prednisone, or azathioprine may be given later, once the disease is under control [97]. The use of azathioprine allows for a lower dose of prednisone, which in turn reduces prednisone's side effects.

In the majority of treated cases disease goes into remission within 1–3 years after starting treatment. Remission occurs when symptoms disappear and laboratory tests show normalization of liver function. Some patients can eventually stop treatment, but their status must carefully be monitored, and the treatment re-instated as soon as the symptoms return. Doses of prednisone or azathioprine may be intermittently necessary for years, if not for life [93,97,98,103]. If standard treatment fails or drug intolerance occurs, alternative therapies can be considered. Patients who are refractory to prednisone or who have severe side effects may benefit from other immunosuppressive agents, such as mycophenylate mofetil, cyclosporine or tacrolimus [93,105]. Insidious forms of autoimmune hepatitis may not be controllable and will progress towards degenerative end-stage liver disease. Such patients should be considered as candidates for liver transplantation.

11.8 Epigenetics of drug-induced liver injury

Risk factors for idiosyncratic drug-induced liver injury include age, sex and genetic polymorphisms of drug-metabolizing enzymes, such as cytochrome P450 and UDP-glucose transferases [4,25,116], which are largely responsible for the formation of drug–protein adducts [41]. However, there are no clear mechanisms to explain a certain level of genetic predisposition in some individuals [116,117].

A possible role of epigenetics to explain differences in susceptibility of humans to chemicals and drugs has recently gained increasing interest [118–120]. Epigenetic mechanisms involve post-synthetic modifications to DNA and/or DNA-associated histones that do not change the DNA sequence itself, but which remodel chromatin and influence gene expression [121]. Increasing evidence suggests that the interactions between genes and the environment very likely play a determinant role in the pathogenesis of allergic diseases, such as asthma, that, despite exhibiting an heritable component, do not follow clear genetic rules. Thus, the same genetic profiles might be associated with different phenotypes depending on which environmental factors may act, influencing the course of an allergic response. This demands a full understanding of the complex gene–environment interactions [121].

In the case of an allergic disease, epigenetics could explain not only the discordances observed between monozygous twins, but also other phenomena that do not conform with genetic premises [120]. Any mechanism proposed to explain the development of a hypersensitivity towards haptens must provide a mechanism for this genetic predisposition, and for the tendency to develop multiple drug sensitivities to (often) completely unrelated compounds. Any theory regarding the mechanism of drug allergy must also explain why, in contrast to what occurs in atopic diseases – that is, allergic rhinitis, asthma and atopic dermatitis – drug allergies, including hepatitis, develop with the same frequency in atopic and non-atopic individuals [20].

An emerging view emphasizes that helper T cells use epigenetic mechanisms to determine the phenotype of their mature cell physiology and actions [122]. During their development, they make sequential cell fate choices to differentiate into Th1 or Th2 cells. All these 'decisions' involve changes in gene expression, and consequently in the phenotype, that are faithfully propagated in an heritable manner to their cellular progeny. With the exception of the TCR, these changes in gene expression occur without a change of the information encoded directly in the DNA sequence. Rather, they appear to be determined through changes in chromatin structure and DNA methylation [123].

There is an association between Th1/Th2 cell immunity and disease, and it is possible that environmentally induced epigenetic changes are involved in Th1/Th2 determination [124]. The most important factor that determines whether a naïve $CD4^+$ T cell differentiates into a Th1 or a Th2 cell is the presence of specific cytokines within the microenvironment in which the naïve T cell is activated. More specifically, Th1 development requires the presence of IL-12, whereas Th2 development requires IL-4. When naive $CD4^+$ T cells differentiate into Th1 or Th2 cells, cytokine loci undergo changes in chromatin structure. The expression of a particular cytokine gene and the silencing of the other seem to be orchestrated by epigenetic mechanisms.

It has been suggested that epigenetic changes of the genes that code for cytokines facilitate selective accessibility of genes in T cells. Cytokine loci undergo changes in chromatin structure when naive $CD4^+$ T cells differentiate into Th1 or Th2 cells. Studies have shown that distal regulatory elements control Th1 (IFN-γ) and Th2 cytokine loci, and are primary targets for tissue-specific transcription factors, serving as centres for epigenetic changes that mark heritable traits in effector cells [125]. In addition, the Th2 response is regulated by the transcription factor GATA3, which is upregulated by signal transducer and activator of transcription 6 (STAT6). The latter is in turn activated by stimulation of the IL-4 receptor in naïve $CD4^+$ cells. In Th2 cells, the increase in GATA3 expression is implicated in the modifications experienced by the gene encoding IL-4, such as DNA methylation and histone acetylation. These phenomena would increase IL-4 gene accessibility and transcription. In addition, IFN-γ silencing would result from DNA methylation and histone deacetylation [120,125].

Finally, recent published data points to toxicants, including pharmaceutical agents, as being capable of altering the epigenetic status of a cell; that is, progressive hypomethylation and aberrant promoter hypermethylation in the liver [126]. This brings new players into the complex scenario of drug-induced liver disease [92].

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References

1. Kaplowitz, N. (2005) Idiosyncratic drug hepatotoxicity. *Nature Reviews Drug Discovery* **4**, 489–499.
2. Zimmerman, H.J. (1999) *Hepatotoxicity: the Adverse Effects of Drugs and Other Chemicals on the Liver*, Lippincott Williams & Wilkins, Philadelphia.
3. Larrey, D. (2000) Drug-induced liver diseases. *Journal of Hepatology* **32**, 77–88.
4. Hussaini, S.H. and Farrington, E.A. (2007) Idiosyncratic drug-induced liver injury: an overview. *Expert Opinion on Drug Safety* **6**, 673–684.
5. Andrade, R.J., Camargo, R., Lucena, M.I. *et al.* (2004) Causality assessment in drug-induced hepatotoxicity. *Expert Opinion on Drug Safety* **3**, 329–344.
6. Lee, W.M. (1995) Drug-induced hepatotoxicity. *New England Journal of Medicine* **333**, 1118–1127.
7. Benichou, C. (1990) Criteria of drug-induced liver disorders. Report of an international consensus meeting. *Journal of Hepatology* **11**, 272–276.
8. Lee, W.M. (2003) Drug-induced hepatotoxicity. *New England Journal of Medicine* **349**, 474–485.
9. Uetrecht, J. (2007) Idiosyncratic drug reactions: current understanding. *Annual Review of Pharmacology and Toxicology* **47**, 513–539.
10. Ju, C. (2005) Immunological mechanisms of drug-induced liver injury. *Current Opinion in Drug Discovery and Development* **8**, 38–43.
11. Watkins, P.B. (2005) Idiosyncratic liver injury: challenges and approaches. *Toxicologic Pathology* **33**, 1–5.
12. Walsh, J.S. (2006) Metabolic activation-role in toxicity and idiosyncratic reactions. In: Borchardt R.T., Kerns E.H., Hageman M.J. *et al.* (eds), *Optimizing the "Drug-Like" Properties of Leads in Drug Discovery*, vol. IV, pp. 50–80. Springer, New York.
13. Maddrey, W.C. (2005) Drug-induced hepatotoxicity: 2005. *Journal of Clinical Gastroenterology* **39**, S83–S89.
14. Cerny, A. and Bertoli, R. (2007) Drug allergic liver injury. In: Pichler W.J. (ed.), *Drug Hypersensitivity*, pp. 278–294. Karger, Basel.
15. Waring, J.F. and Anderson, M.G. (2005) Idiosyncratic toxicity: mechanistic insights gained from analysis of prior compounds. *Current Opinion in Drug Discovery and Development* **8**, 59–65.
16. Castell, J.V. (1998) Allergic hepatitis: a drug-mediated organ-specific, immune reaction. *Clinical and Experimental Allergy* **28**(S4), 13–19.
17. Markel, A. (2005) Allopurinol-induced DRESS syndrome. *Israel Medical Association Journal* **7**, 656–660.
18. Liguori, M.J. and Waring, J.F. (2006) Investigations toward enhanced understanding of hepatic idiosyncratic drug reactions. *Expert Opinion on Drug Metabolism and Toxicology* **2**, 835–846.
19. Watkins, P.B. and Seeff, L.B. (2006) Drug-induced liver injury: summary of a single topic clinical research conference. *Hepatology* **43**, 618–631.
20. Borish, L. and Tilles, S.A. (1998) Immune mechanisms of drug allergy. *Immunology and Allergy Clinics of North America* **18**, 717–729.
21. Podevin, P. and Biour, M. (1995) Drug-induced "allergic hepatitis". *Clinical Reviews in Allergy and Immunology* **13**, 223–244.

22. Castell, J.V. and Castell, M. (2006) Allergic hepatitis induced by drugs. *Current Opinion in Allergy and Clinical Immunology* **6**, 258–265.
23. Posadas, S.J. and Pichler, W.J. (2007) Delayed drug hypersensitivity reactions - new concepts. *Clinical and Experimental Allergy* **37**, 989–999.
24. Gomez-Lechon, M.J., Carrasquer, J., Berenguer, J. *et al.* (1996) Evidence of antibodies to erythromycin in serum of a patient following an episode of acute drug-induced hepatitis. *Clinical and Experimental Allergy* **26**, 590–596.
25. Obermayer-Straub, P., Strassburg, C.P. and Manns, M.P. (2000) Target proteins in human autoimmunity: cytochromes P450 and UDP- glucuronosyltransferases. *Canadian Journal of Gastroenterology* **14**, 429–439.
26. Njoku, D.B., Mellerson, J.L., Talor, M.V. *et al.* (2006) Role of CYP2E1 immunoglobulin G4 subclass antibodies and complement in pathogenesis of idiosyncratic drug-induced hepatitis. *Clinical and Vaccine Immunology* **13**, 258–265.
27. Liu, Z.X. and Kaplowitz, N. (2002) Immune-mediated drug-induced liver disease. *Clinics in Liver Disease* **6**, 755–774.
28. Neuberger, J., Kenna, J.G., Nouri Aria, K. *et al.* (1985) Antibody mediated hepatocyte injury in methyl dopa induced hepatotoxicity. *Gut* **26**, 1233–1239.
29. Pichler, W.J. (2003) Delayed drug hypersensitivity reactions. *Annals of Internal Medicine* **139**, 683–693.
30. Pichler, W.J. (2003) Drug-induced autoimmunity. *Current Opinion in Allergy and Clinical Immunology* **3**, 249–253.
31. Uetrecht, J. (2005) Role of drug metabolism for breaking tolerance and the localization of drug hypersensitivity. *Toxicology* **209**, 113–118.
32. Boelsterli, U.A. (1993) Specific targets of covalent drug-protein interactions in hepatocytes and their toxicological significance in drug-induced liver injury. *Drug Metabolism Reviews* **25**, 395–451.
33. Walgren, J.L., Mitchell, M.D. and Thompson, D.C. (2005) Role of metabolism in drug-induced idiosyncratic hepatotoxicity. *Critical Reviews in Toxicology* **35**, 325–361.
34. Park, B.K., Kitteringham, N.R., Maggs, J.L. *et al.* (2005) The role of metabolic activation in drug-induced hepatotoxicity. *Annual Review of Pharmacology and Toxicology* **45**, 177–202.
35. Sanderson, J.P., Naisbitt, D.J. and Park, B.K. (2006) Role of bioactivation in drug-induced hypersensitivity reactions. *American Association of Pharmaceutical Scientists Journal* **8**, E55–E64.
36. Naisbitt, D.J., Williams, D.P., Pirmohamed, M. *et al.* (2001) Reactive metabolites and their role in drug reactions. *Current Opinion in Allergy and Clinical Immunology* **1**, 317–325.
37. Baldo, B.A., Pham, N.H. and Zhao, Z. (2001) Chemistry of drug allergenicity. *Current Opinion in Allergy and Clinical Immunology* **1**, 327–335.
38. Guengerich, F.P. (2005) Principles of covalent binding of reactive metabolites and examples of activation of bis-electrophiles by conjugation. *Archives of Biochemistry and Biophysics* **433**, 369–378.
39. Gil, M.L., Ramirez, M.C., Terencio, M.C. *et al.* (1995) Immunochemical detection of protein adducts in cultured human hepatocytes exposed to diclofenac. *Biochimica et Biophysica Acta* **1272**, 140–146.
40. Pumford, N.R., Halmes, N.C. and Hinson, J.A. (1997) Covalent binding of xenobiotics to specific proteins in the liver. *Drug Metabolism Reviews* **29**, 39–57.
41. Evans, D.C., Watt, A.P., Nicoll-Griffith, D.A. *et al.* (2004) Drug-protein adducts: an industry perspective on minimizing the potential for drug bioactivation in drug discovery and development. *Chemical Research in Toxicology* **17**, 3–16.
42. Cohen, S.D., Pumford, N.R., Khairallah, E.A. *et al.* (1997) Selective protein covalent binding and target organ toxicity. *Toxicology and Applied Pharmacology* **143**, 1–12.

43. Park, B.K., Naisbitt, D.J., Gordon, S.F. *et al.* (2001) Metabolic activation in drug allergies. *Toxicology* **158**, 11–23.
44. Knowles, S.R., Shapiro, L.E. and Shear, N.H. (2003) Reactive metabolites and adverse drug reactions: clinical considerations. *Clinical Reviews in Allergy and Immunology* **24**, 229–238.
45. Matzinger, P. (2002) The danger model: a renewed sense of self. *Science* **296**, 301–305.
46. Pirmohamed, M., Naisbitt, D.J., Gordon, F. *et al.* (2002) The danger hypothesis–potential role in, idiosyncratic drug reactions. *Toxicology* **181–182**, 55–63.
47. Seguin, B. and Uetrecht, J. (2003) The danger hypothesis applied to idiosyncratic drug reactions. *Current Opinion in Allergy and Clinical, Immunology* **3**, 235–242.
48. Pichler, W.J. (2005) Direct T-cell stimulations by drugs bypassing the innate immune system. *Toxicology* **209**, 95–100.
49. Roujeau, J.C. (2006) Immune mechanisms in drug allergy. *Allergology International* **55**, 27–33.
50. Knolle, P.A. and Gerken, G. (2000) Local control of the immune response in the liver. *Immunological Reviews* **174**, 21–34.
51. Longhi, M.S., Ma, Y., Mitry, R.R. *et al.* (2005) Effect of CD4 + CD25 + regulatory T-cells on CD8 T-cell function in patients with autoimmune hepatitis. *Journal of Autoimmunity* **25**, 63–71.
52. Trauner, M., Meier, P.J. and Boyer, J.L. (1998) Molecular pathogenesis of cholestasis. *New England Journal of Medicine* **339**, 1217–1227.
53. Racanelli, V. and Rehermann, B. (2006) The liver as an immunological organ. *Hepatology* **43**, S54–S62.
54. Bowen, D.G., Zen, M., Holz, L. *et al.* (2004) The site of primary T cell activation is a determinant of the balance between intrahepatic tolerance and immunity. *Journal of Clinical Investigation* **114**, 701–712.
55. Ju, C. and Pohl, L.R. (2005) Tolerogenic role of Kupffer cells in immune-mediated adverse drug reactions. *Toxicology* **209**, 109–112.
56. Knolle, P.A. (2006) Involvement of the liver in the induction of CD8 T cell tolerance towards oral antigen. *Zeitschrift für Gastroenterologie* **44**, 51–56.
57. Onoe, T., Ohdan, H., Tokita, D. *et al.* (2005) Liver sinusoidal endothelial cells tolerize T cells across MHC barriers in mice. *Journal of Immunology* **175**, 139–146.
58. Farrelly, C. and Doherty, D.G. (2007) A short primer on fundamental immunology. In: Gershwin, M.E., Vierling, J.M. and Manns, M.P. (eds), *Liver Immunology. Principles and Practice*, pp. 15–24. Humana Press, Totowa, NJ.
59. Sumpter, T.L., Abe, M., Tokita, D. *et al.* (2007) Dendritic cells, the liver, and transplantation. *Hepatology* **46**, 2021–2031.
60. Hsu, W., Shu, S.A., Gershwin, E. *et al.* (2007) The current immune function of hepatic dendritic cells. *Cellular and Molecular Immunology* **4**, 321–328.
61. Parker, G.A. and Picut, C.A. (2005) Liver immunobiology. *Toxicologic Pathology* **33**, 52–62.
62. Abe, M. and Thomson, A.W. (2007) Antigen processing and presentation in the liver. In: Gershwin, M.E., Vierling, J.M. and Manns, M.P. (eds), *Liver Immunology. Principles and Practice*, pp. 49–60. Humana Press, Totowa, NJ.
63. Lau, A.H. and Thomson, A.W. (2003) Dendritic cells and immune regulation in the liver. *Gut* **52**, 307–314.
64. Crispe, I.N. (2003) Hepatic T cells and liver tolerance. *Nature Reviews Immunology* **3**, 51–62.
65. Bowen, D.G., McCaughan, G.W. and Bertolino, P. (2005) Intrahepatic immunity: a tale of two sites? *Trends in Immunology* **26**, 512–517.
66. Masson, M.J., Peterson, R.A., Chung, C.J. *et al.* (2007) Lymphocyte loss and immunosuppression following acetaminophen-induced hepatotoxicity in mice as a potential mechanism of tolerance. *Chemical Research in Toxicology* **20**, 20–26.

67. Rubinstein, D., Roska, A.K. and Lipsky, P.E. (1987) Antigen presentation by liver sinusoidal lining cells after antigen exposure in vivo. *Journal of Immunology* **138**, 1377–1382.
68. Lohse, A.W., Knolle, P.A., Bilo, K. *et al.* (1996) Antigen-presenting function and B7 expression of murine sinusoidal endothelial cells and Kupffer cells. *Gastroenterology* **110**, 1175–1181.
69. Squiers, E.C., Brunson, M.E. and Salomon, D.R. (1993) Kupffer cells can present alloantigen in vitro: an effect abrogated by gadolinium. *Journal of Surgical Research* **55**, 571–574.
70. Christen, U., Burgin, M. and Gut, J. (1991) Halothane metabolism: Kupffer cells carry and partially process trifluoroacetylated protein adducts. *Biochemical and Biophysical Research Communications* **175**, 256–262.
71. Bertolino, P., McCaughan, G.W. and Bowen, D.G. (2002) Role of primary intrahepatic T-cell activation in the 'liver tolerance effect'. *Immunology and Cell Biology* **80**, 84–92.
72. Maemura, K., Zheng, Q., Wada, T. *et al.* (2005) Reactive oxygen species are essential mediators in antigen presentation by Kupffer cells. *Immunology and Cell Biology* **83**, 336–343.
73. Prandota, J. (2005) Important role of proinflammatory cytokines/other endogenous substances in drug-induced hepatotoxicity: depression of drug metabolism during infections/inflammation states, and genetic polymorphisms of drug-metabolizing enzymes/cytokines may markedly contribute to this pathology. *American Journal of Therapeutics* **12**, 254–261.
74. Ju, C., McCoy, J.P., Chung, C.J. *et al.* (2003) Tolerogenic role of Kupffer cells in allergic reactions. *Chemical Research in Toxicology* **16**, 1514–1519.
75. You, Q., Cheng, L. and Ju, C. (2007) Role of hepatic Kupffer cells in inducing T cell tolerance. *Journal of Immunology* **178**, 128.12.
76. Roberts, R.A., Ganey, P.E., Ju, C. *et al.* (2007) Role of the Kupffer cell in mediating hepatic toxicity and carcinogenesis. *Toxicological Sciences* **96**, 2–15.
77. Bowen, D.G., Zen, M., Holz, L. *et al.* (2004) The site of primary T cell activation is a determinant of the balance between intrahepatic tolerance and immunity. *Journal of Clinical Investigation* **114**, 701–712.
78. Villadangos, J.A., Schnorrer, P. and Wilson, N.S. (2005) Control of MHC class II antigen presentation in dendritic cells: a balance between creative and destructive forces. *Immunological Reviews* **207**, 191–205.
79. Usharauli, D. (2005) Dendritic cells and the immunity/tolerance decision. *Medical Hypotheses* **64**, 112–113.
80. Tan, J.K. and O'Neill, H.C. (2005) Maturation requirements for dendritic cells in T cell stimulation leading to tolerance versus immunity. *Journal of Leukocyte Biology* **78**, 319–324.
81. Rock, K.L. and Shen, L. (2005) Cross-presentation: underlying mechanisms and role in immune surveillance. *Immunological Reviews* **207**, 166–183.
82. Maldonado-Lopez, R. and Moser, M. (2001) Dendritic cell subsets and the regulation of Th1/Th2 responses. *Seminars in Immunology* **13**, 275–282.
83. Arvieux, C., Calmus, Y., Gane, P. *et al.* (1993) Immunogenicity of rat hepatocytes in vivo: effect of cholestasis-induced changes in major histocompatibility complex expression. *Journal of Hepatology* **18**, 335–341.
84. Franco, A., Barnaba, V., Natali, P. *et al.* (1988) Expression of class I and class II major histocompatibility complex antigens on human hepatocytes. *Hepatology* **8**, 449–454.
85. Speeti, M., Stahls, A., Meri, S. *et al.* (2003) Upregulation of major histocompatibility complex class II antigens in hepatocytes in Doberman hepatitis. *Veterinary Immunology and Immunopathology* **96**, 1–12.
86. Herkel, J., Jagemann, B., Wiegard, C. *et al.* (2003) MHC class II-expressing hepatocytes function as antigen-presenting cells and activate specific CD4 T lymphocyutes. *Hepatology* **37**, 1079–1085.

87. Tsutsui, H., Terano, Y., Sakagami, C. *et al.* (1992) Drug-specific T cells derived from patients with drug-induced allergic hepatitis. *Journal of Immunology* **149**, 706–716.
88. Liu, Z.X., Govindarajan, S. and Kaplowitz, N. (2004) Innate immune system plays a critical role in determining the progression and severity of acetaminophen hepatotoxicity. *Gastroenterology* **127**, 1760–1774.
89. Miyakawa, R., Ichida, T., Yamagiwa, S. *et al.* (2005) Hepatic natural killer and natural killer T cells markedly decreased in two cases of drug-induced fulminant hepatic failure rescued by living donor liver transplantation. *Journal of Gastroenterology and Hepatology* **20**, 1126–1130.
90. Leist, M., Gantner, F., Jilg, S. *et al.* (1995) Activation of the 55 kDa TNF receptor is necessary and sufficient for TNF-induced liver failure, hepatocyte apoptosis, and nitrite release. *Journal of Immunology* **154**, 1307–1316.
91. Ju, C., Reilly, T.P., Bourdi, M. *et al.* (2002) Protective role of Kupffer cells in acetaminophen-induced hepatic injury in mice. *Chemical Research in Toxicology* **15**, 1504–1513.
92. Lohse, A.W. and Hennes, E. (2007) Diagnostic criteria for autoimmune hepatitis. *Hepatlogy Research* **37** (suppl 3), S509.
93. Krawitt, E.L. (2006) Autoimmune hepatitis. *New England Journal of Medicine* **354**, 54–66.
94. Khokhar, O., Gange, C., Clement, S. *et al.* (2005) Autoimmune hepatitis and thyroiditis associated with rifampin and pyrazinamide prophylaxis: an unusual reaction. *Digestive Diseases and Sciences* **50**, 207–211.
95. van Heyningen, C. (2005) Drug-induced acute autoimmune hepatitis during combination therapy with atorvastatin and ezetimibe. *Annals of Clinical Biochemistry* **42**, 402–404.
96. Abe, M., Furukawa, S., Takayama, S. *et al.* (2003) Drug-induced hepatitis with autoimmune features during minocycline therapy. *Annals of Internal Medicine* **42**, 48–52.
97. Abraham, C., Hart, J., Locke, S.M. *et al.* (2008) A case of indometacin-induced acute hepatitis developing into chronic autoimmune hepatitis. *Nature Clinical Practice Gastroenterology &, Hepatology* **5**, 172–176.
98. Alla, V., Abraham, J., Siddiqui, J. *et al.* (2006) Autoimmune hepatitis triggered by statins. *Journal of Clinical Gastroenterology* **40**, 757–761.
99. Anderson, J.S., Rose, N.R., Martin, J.L. *et al.* (2007) Desflurane hepatitis associated with hapten and autoantigen-specific IgG4 antibodies. *Anesthesia and Analgesia* **104**, 1452–1453.
100. Chamberlain, M.C., Schwarzenberg, S.J., Akin, E.U. *et al.* (2006) Minocycline-induced autoimmune hepatitis with subsequent cirrhosis. *Journal of Pediatric Gastroenterology and Nutrition* **42**, 232–235.
101. Csepregi, A., Treiber, G., Rocken, C. *et al.* (2005) Acute exacerbation of autoimmune hepatitis induced by Twinrix. *World Journal of Gastroenterology* **11**, 4114–4116.
102. Germano, V., Picchianti Diamanti, A., Baccano, G. *et al.* (2005) Autoimmune hepatitis associated with infliximab in a patient with psoriatic arthritis. *Annals of the Rheumatic Diseases* **64**, 1519–1520.
103. Graziadei, I.W., Obermoser, G.E., Sepp, N.T. *et al.* (2003) Drug-induced lupus-like syndrome associated with severe autoimmune hepatitis. *Lupus* **12**, 409–412.
104. Kim, S.Y., Kim, B.H., Dong, S.H. *et al.* (2007) Alfuzosin-induced acute liver injury. *Korean Journal of Hepatology* **13**, 414–418.
105. Lewis, J.L. and Zimmerman, H.J. (1998) Drug-induced autoimmune liver disease. In: Krawitt, E. L., Wiesner, R.H. and Nishioka, M. (eds), *Autoimmune Liver Diseases*, pp. 627–649. Elsevier, Amsterdam.
106. Pelli, N. and Setti, M. (2004) Atorvastatin as a trigger of autoimmune hepatitis. *Journal of Hepatology* **40**, 716.

107. Ozorio, G., McGarity, B., Bak, H. *et al.* (2007) Autoimmune hepatitis following infliximab therapy for ankylosing spondylitis. *Medical Journal of Australia* **187**, 524–526.
108. Pompili, M., Basso, M., Grieco, A. *et al.* (2004) Recurrent acute hepatitis associated with use of cetirizine. *Annals of Pharmacotherapy* **38**, 1844–1847.
109. Wolters, L.M. and Van Buuren, H.R. (2005) Rosuvastatin-associated hepatitis with autoimmune features. *European Journal of Gastroenterology and Hepatology* **17**, 589–590.
110. Rubin, R.L. (2005) Drug-induced lupus. *Toxicology* **209**, 135–147.
111. Lewis, J.L. (2000) Drug-induced liver disease. *Medical Clinics of North America* **84**, 1275–1311.
112. Manns, M.P. and Vogel, A. (2006) Autoimmune hepatitis, from mechanisms to therapy. *Hepatology* **43**, S132–S144.
113. Anderson, M.S., Venanzi, E.S., Chen, Z. *et al.* (2005) The cellular mechanism of Aire control of T cell tolerance. *Immunity* **23**, 227–239.
114. Vogel, A., Liermann, H., Harms, A. *et al.* (2001) Autoimmune regulator AIRE: evidence for genetic differences between autoimmune hepatitis and hepatitis as part of the autoimmune polyglandular syndrome type 1. *Hepatology* **33**, 1047–1052.
115. Montano Loza, A.J. and Czaja, A.J. (2007) Current therapy for autoimmune hepatitis. *Nature Clinical Practice Gastroenterology & Hepatology* **4**, 202–214.
116. Larrey, D. (2002) Epidemiology and individual susceptibility to adverse drug reactions affecting the liver. *Seminars in Liver Disease* **22**, 145–155.
117. Ermann, J. and Fathman, C.G. (2001) Autoimmune diseases: genes, bugs and failed regulation. *Nature Immunology* **2**, 759–761.
118. Cookson, W. (1999) The alliance of genes and environment in asthma and allergy. *Nature* **402**, B5–B11.
119. Rautajoki, K.J., Kylaniemi, M.K., Raghav, S.K. *et al.* (2008) An insight into molecular mechanisms of human T helper cell differentiation. *Annals of Medicine* **40**, 322–335.
120. Isidoro-Garcia, M., Davila-Gonzalez, I., Pascual de Pedro, M. *et al.* (2007) Interactions between genes and the environment. Epigenetics in allergy. *Allergologia et Immunopathologia* **35**, 254–258.
121. Vercelli, D. (2004) Genetics, epigenetics, and the environment: switching, buffering, releasing. *Journal of Allergy and Clinical Immunology* **113**, 381–386.
122. Reiner, S.L. (2005) Epigenetic control in the immune response. *Human Molecular Genetics* **14**, R41–R46.
123. Wilson, C.B., Makar, K.W. and Perez-Melgosa, M. (2002) Epigenetic regulation of T cell fate and function. *Journal of Infectious Diseases* **185** (suppl 1), S37–S45.
124. Sanders, V.M. (2006) Epigenetic regulation of Th1 and Th2 cell development. *Brain, Behavior and Immunity* **20**, 317–324.
125. Lee, G.R., Kim, S.T., Spilianakis, C.G. *et al.* (2006) T helper cell differentiation: regulation by cis elements and epigenetics. *Immunity* **24**, 369–379.
126. Kovalenko, V.M., Bagnyukova, T.V., Sergienko, O.V. *et al.* (2007) Epigenetic changes in the rat livers induced by pyrazinamide treatment. *Toxicology and Applied Pharmacology* **225**, 293–299.
127. Shalom, R., Rimbroth, S., Rozenman, D. *et al.* (2008) Allopurinol-induced recurrent DRESS syndrome: pathophysiology and treatment. *Renal Failure* **30**, 327–329.
128. Syn, W.K., Naisbitt, D.J., Holt, A.P. *et al.* (2005) Carbamazepine-induced acute liver failure as part of the DRESS syndrome. *International Journal of Clinical Practice* **59**, 988–991.
129. Itha, S., Kumar, A., Dhingra, S. *et al.* (2003) Dapsone induced cholangitis as a part of dapsone syndrome: a case report. *BMC Gastroenterology* **3**, 21.
130. Agrawal, S. and Agarwalla, A. (2005) Dapsone hypersensitivity syndrome: a clinico-epidemiological review. *Journal of Dermatology* **32**, 883–889.

131. Frouin, E., Roth, B., Grange, A. *et al.* (2005) Hypersensitivity to fluindione (Previscan). Positive skin patch tests. *Annales de dermatologie et de venereologie* **132**, 1000–1002.
132. Basset, C., Vadrot, J., Denis, J. *et al.* (2003) Prolonged cholestasis and ductopenia following gold salt therapy. *Liver International* **23**, 89–93.
133. Mecarelli, O., Pulitano, P., Mingoia, M. *et al.* (2005) Acute hepatitis associated with lamotrigine and managed with the molecular adsorbents recirculating system (Mars). *Epilepsia* **46**, 1687–1689.
134. Herdeg, C., Hilt, F., Buchtemann, A. *et al.* (2002) Allergic cholestatic hepatitis and exanthema induced by metamizole: verification by lymphocyte transformation test. *Liver* **22**, 507–513.
135. Knudtson, E., Para, M., Boswell, H. *et al.* (2003) Drug rash with eosinophilia and systemic symptoms syndrome and renal toxicity with a nevirapine-containing regimen in a pregnant patIent with human immunodeficiency virus. *Obstetrics & Gynecology* **101**, 1094–1097.
136. Claes, P., Wintzen, M., Allard, S. *et al.* (2004) Nevirapine-induced toxic epidermal necrolysis and toxic hepatitis treated successfully with a combination of intravenous immunoglobulins and N-acetylcysteine. *European Journal of Internal Medicine* **15**, 255–258.
137. Maniar, J.K., Shah, S.R., Verma, R. *et al.* (2006) Nevirapine-induced fulminant hepatitis. *Journal of Association of Physicians of India* **54**, 957–958.
138. Torti, C., Costarelli, S., De Silvestri, A. *et al.* (2007) Analysis of severe hepatic events associated with nevirapine-containing regimens: CD4 + T-cell count and gender in hepatitis C seropositive and seronegative patients. *Drug Safety* **30**, 1161–1169.
139. Onur, O., Guneysel, O., Denizbasi, A. *et al.* (2007) Acute hepatitis attack after exposure to telithromycin. *Clinical Therapeutics* **29**, 1725–1729.

Abstract

Drug-induced allergic hepatitis is a liver-specific inflammatory reaction resulting from a hypersensitivity to a particular drug. Clinically, both hepatocellular injury and cholestasis can occur, and most episodes have a good clinical prognosis upon drug withdrawal. Allergic hepatitis induced by drugs is a type IV hypersensitivity reaction that involves $CD4^+CD8^+$ cytotoxic lymphocytes as well as natural killer cells. However, antibodies directed to the drug are less common, and their pathogenic role is not clear. In some cases, damage to liver cells may continue in the form of an autoimmune hepatitis. A classical pathogenic mechanism identifies formation of drug–protein adducts and presentation, and/or their direct interaction with the major histocompatibility complex–T cell receptor complex as an *initiating* factor that triggers a hypersensitivity reaction. In the past few years, epigenetics has gained attention as a possible factor that could explain differences in susceptibility of humans to drugs and other chemicals.

Key words: autoimmune hepatitis; drug-induced allergic hepatitis; drug-induced liver injury; drug hepatotoxicity; hepatocytes; idiosyncratic hepatotoxicity; T cell; tolerance.

PART III

Epigenetic Modifiers of Autoimmunity

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12

Epigenetic modifications associated with T cell tolerance

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12.1 Immunity versus tolerance

The vertebrate immune system has evolved to protect against invasive pathogens, but with this adaptation comes the risk of autoimmunity, the recognition and destruction of self tissues by cells of the immune system. To avoid this problem the system has evolved active mechanisms by which tolerance is induced against self-antigens. These mechanisms include deletion of autoreactive lymphocytes, functional inactivation of lymphocytes (anergy) and suppression of inflammatory responses by a specialized subset of regulatory T cells (Tregs). The latter two modes of tolerance induction involve active transcriptional repression of pro-inflammatory cytokine genes in the affected T lymphocytes, and are likely mediated via epigenetic mechanisms. Therefore, anergy affecting the expression of the *il2* and *ifnγ* genes in conventional T cells and Tregs will serve as the main subject of discussion in this review.

12.2 Epigenetic regulation of the physical structure of genomic DNA

In eukaryotic cells activation of transcription factors is often not sufficient for gene transcription. The DNA that constitutes each chromosome is wrapped around octamers of histone proteins called nucleosomes, which are in turn incorporated into higher-order structures that allow efficient packaging of the approximately 3 billion

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nucleotides of the genome into each cell's nucleus [10]. The physical structure of chromatin is dynamic, and can be influenced by the chemical modification of the histones, the basic unit of the nucleosome, by enzymes that attach functional groups to defined amino acids within histone 'tails' [11,12]. Acetylation by histone acetyl transferases (HATs) inhibits nucleosome compaction, and generates binding sites for ATP-dependent chromatin-remodelling enzymes that loosen and slide the DNA from around the nucleosomes [13]. This renders regulatory regions accessible to DNA-binding factors, and in this way histone acetylation promotes local gene transcription. Conversely, methylation by histone methyltransferases generates binding sites for histone deacetylases (HDACs) and DNA methyltransferases, which oppose the action of the HATs and act to inhibit local gene transcription [14,15]. DNA methylation can also influence the binding of transcription factors to gene promoters. Methylation of cytosine/guanine (CpG) dinucleotides within *cis* elements can impose a direct physical constraint to transcription factor binding. In addition, DNA methylation forms binding sites for methylated CpG-binding proteins (MeCPs or MBDs), which in turn recruit HDACs and histone methyltransferases to the local chromatin [16–18]. In the end, whether a particular gene is wrapped tightly around a nucleosome, and whether the nucleosomes are in turn packed together loosely or tightly, can have a major influence on its expression.

Although large-scale chromatin structure can have a major influence over gene expression, nucleosome remodelling is not necessarily required for the transcriptional activation of all genes. The requirement for remodelling is thought to be determined by the local positioning of nucleosomes with respect to the TATA box and transcriptional start site at a given gene; that is, if a nucleosome is positioned over the transcriptional start site of a gene, chromatin-remodelling factors will probably be required to reposition the nucleosome before transcription will occur [19]. Conversely, if the start site is not occupied by a nucleosome under non-inducing conditions, then chromatin remodelling may not be required for transcription to proceed. Classical examples of these two types of loci are the genes that encode interferon (IFN)-β and interleukin (IL)-8. The IL-8 gene contains a nucleosome positioned well downstream of the TATA box and the transcriptional start site [19]. The promoter is constitutively occupied by CCAAT/enhancer-binding protein (C/EBP) β, and requires only the inducible binding of nuclear factor κB (NF-κB) for full gene expression [20]. Conversely, the IFN-β gene contains a nucleosome positioned over the TATA box and the transcriptional start site, where it blocks assembly of the pre-initiation complex [21]. The IFN-β promoter/enhancer spans approximately 70 bp, and contains multiple *cis* elements that mediate the cooperative binding of several transcription factors, including activating transcription factor (ATF), c-Jun, IRF-1 and NF-κB [22,23]. In addition, the IFN-β promoter/enhancer contains several important high-mobility group (HMG) protein HMG I(Y)-binding sites. HMG I(Y) does not directly mediate transcriptional activation, but functions to induce DNA bending that is thought to facilitate the folding back of distal elements into closer proximity to the TATA box. This specific architecture of the IFN-β promoter/enhancer, which has been termed the enhanceosome, is crucial for IFN-β gene expression, because an IL-8 promoter artificially spliced to the IFN-β

gene is unable to recruit histone-modifying enzymes, induces chromatin remodelling and drives transcription in response to the same signals that would allow expression of the IL-8 gene [19]. Upon stimulation of gene expression, the ATF, c-Jun, IRF-1 and NF-κB transcription factors bind cooperatively to the IFN-β promoter/enhancer and recruit HATs (general control non-derepressible (GCN) 5 and p300/cAMP-response-element-binding protein (CREB)-binding protein (CBP)) that acetylate the positioned nucleosome. Histone acetylation results in recruitment of the SWI/SNF chromatin-remodelling complex, which slides the nucleosome away from the transcriptional start site. This opens the TATA box and results in binding of TATA box-binding protein (TBP) and TFIID transcription factors, and RNA polymerase, and initiation of transcription.

12.3 Epigenetic control of pro-inflammatory cytokine gene transcription

The structure and regulation of the *il2* and *ifn*γ genes in T cells resembles that of the IFN-β enhanceosome in many aspects, and multiple lines of evidence suggest that the expression of these genes is under epigenetic control. For instance, both genes are controlled by the concerted action of a promoter and one or more enhancers (at least six in the case of the *ifn*γ locus) [24–27]. In addition, proper tissue-specific and signal-dependent expression of the *il2* gene depends upon a locus control region located approximately 8 kb upstream of the transcriptional start site, suggesting a specific role for local chromatin context [28]. Indeed, this region exhibits stimulus-independent methylation on Lys-4 of histone H3 (dimethylH3K4), a relatively stable histone modification associated with non-silenced and transcriptionally active genes [29], in cells capable of producing IL-2 [30]. The *il2* and *ifn*γ loci in quiescent T cells contain hypo-acetylated nucleosomes (Figure 12.1) positioned throughout the promoter region and the transcriptional start site that block access to transcription factors [28,31–39]. Crucial regulatory regions of the *il2* promoter/enhancer such as the ARRE-1, ARRE-2 and CD28RE are also occupied by HMG I(Y) architectural DNA-bending proteins, which are required for normal expression of the gene [40,41], and the CD28RE of the *ifn*γ promoter likewise contains a conserved HMG I(Y) site. Genomic DNA is fully methylated at CpG dinucleotides within the *ifn*γ intronic enhancer in naive $CD4^+$ and $CD8^+$ T cells, while the promoter region is less than 25% methylated in both subsets [35,39,42–48]. The *il2* promoter/enhancer region shows a similar pattern of methylation as the *ifn*γ promoter, with 25–35% of the CpG dinucleotides exhibiting methylation in $CD4^+$ T cells, whereas this promoter is 35–45% methylated in $CD8^+$ T cells, which produce IL-2 less efficiently than $CD4^+$ T cells [35,39,42,43].

Upon productive T cell activation, the promoter and enhancer regions of the *il2* and *ifn*γ genes exhibit rapid histone acetylation and nucleosome remodelling (Figure 12.1) [28,31–39,49,50]. This is accompanied by the cooperative binding of transcription factors such as Oct-1, NF-κB/c-Rel, nuclear factor of activated T cells

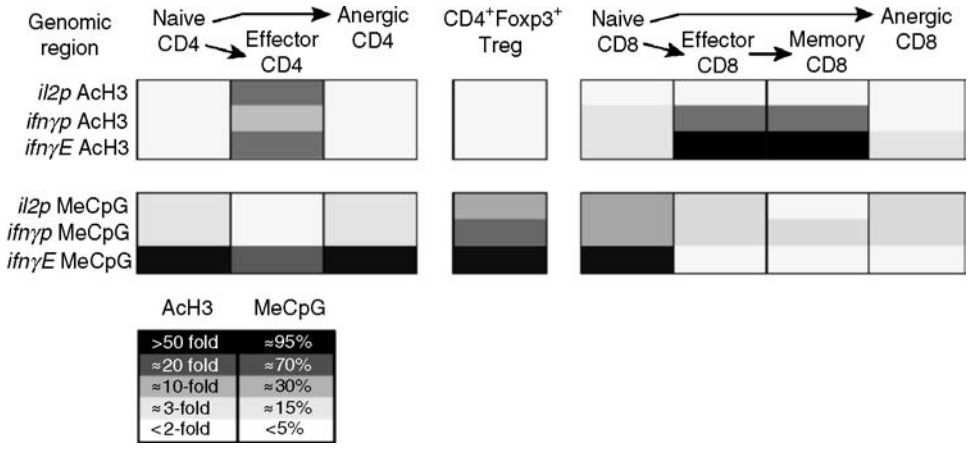

Figure 12.1 The chart depicts the relative degree of histone acetylation (AcH3) and DNA methylation (MeCpG) at the *il2* promoter (*il2p*), *ifnγ* promoter (*ifnγp*) and *ifnγ* enhancer (*ifnγE*) in three T cell lineages: $CD4^+$, $CD8^+$ and $Foxp3^+$ Tregs. Epigenetic changes are also tracked as a function of the induction of anergy versus effector/memory differentiation from naïve precursors. As shown in the key, darker shades indicate more acetylation or methylation.

(NFAT), CREB and activator protein 1 (AP-1) to defined elements within the promoter-enhancer, with additional factors such as T-bet and signal transducer and activator of transcription 4 (STAT4) being required for expression of the *ifnγ* gene [24–27,51,52]. NF-κB, NFAT, CREB and AP-1 are each able to bind HAT co-activators such as p300/CBP in T cells [53,54], and CBP is specifically recruited to the *il2* promoter in Jurkat cells and $CD4^+$ T cells in response to T cell receptor (TCR)/CD28 co-stimulation [55–57]. In addition, the *ifnγ* locus exhibits an activation-dependent increase in dimethylH3K4 [7]. The SWI/SNF-like BAF nucleosome-remodelling complex is recruited to chromatin in response to TCR signalling in T cells [58], and is likely to contribute to chromatin remodelling at cytokine gene loci. Like the IFN-β enhanceosome, large-scale intrachromosomal looping occurs in $CD4^+$ T cells at the *il4* and *ifnγ* loci, resulting in physical association between the promoter and distant locus control region/enhancer elements [59,60]. Productive activation also favours demethylation of regulatory DNA elements at the *il2* and *ifnγ* loci in $CD4^+$ T cells (particularly during T helper (Th) 1 differentiation) and in memory $CD8^+$ T cells [35,39,42,43,61].

Are these epigenetic changes important for the expression of these cytokine genes? Several lines of evidence indicate that this is the case. Global inhibition of DNA methylation during T cell activation leads to failed gene silencing and promiscuous IFN-γ, IL-2 and IL-4 production [62–64], and site-specific methods of modulating DNA methylation have been used to demonstrate that methylation of distinct CpG dinucleotides in the *ifnγ* and *il2* promoters inhibits the expression of these genes in $CD4^+$ T cells [61,65,66]. Similarly, global inhibition of HDAC activity has been shown to promote expression of the *ifnγ* and *il4* genes [67,68]. These epigenetic mechanisms

may explain previous observations that the *il2* gene under some circumstances exhibits a monoallelic pattern of expression, such that an individual T cell 'chooses' which one of the two alleles to express, and silences the other [69,70]. This choice is stably inherited by the progeny of the cell following cell division. Also, the *ifnγ* gene is not expressed in differentiating T cells until four to five cell divisions have been achieved [45,71,72]. Likewise, the frequency of T cells that produce IL-2 after re-stimulation is also coupled to the number of cell divisions achieved during the primary stimulus [72–74], suggesting that cell division opposes mechanisms that keep these loci closed following initial antigen encounter.

12.4 Epigenetic silencing of cytokine genes in tolerant T cells

Activation of conventional $CD4^+$ and $CD8^+$ T lymphocytes in the absence of the appropriate co-stimulatory signals results in clonal anergy [75]. Anergy refers to a specific situation in which antigen-specific T cells that have been exposed to a stimulus are present (i.e. have not been deleted), but exist in a state of reduced function or hypo-responsiveness [75]. $CD4^+$ T cell anergy occurs when the cells fail to receive CD28 co-stimulation [76], whereas $CD8^+$ T cells undergo transient hypo-responsiveness as a normal consequence of primary stimulation [77]. However, high-dose or chronic antigenic stimulation, lack of co-stimulation or cytokines, or lack of $CD4^+$ T cell help can lead to a stable state of $CD8^+$ anergy [77–79]. A hallmark of anergy, as first described in $CD4^+$ T helper clones, is the failure to proliferate in response to re-stimulation through the TCR and CD28, due to an inability to synthesize the major T cell growth factor IL-2 [75]. Primary anergic $CD4^+$ and $CD8^+$ T cells also have a severe defect in the production of inflammatory cytokines such as IFN-γ [72,78,80]. This phenotype is associated with enhanced expression of E3 ubiquitin ligases and proteolytic degradation of signalling molecules [81], which probably contributes to the altered recruitment of TCR-associated enzymes [82–84] and defective activation of mitogen-activated protein kinase (MAPK) cascades observed in anergic cells [74,82,85–89]. Similar proximal defects in TCR signalling have been observed in $Foxp3^+CD4^+$ regulatory T cells (Tregs) and anergic $CD8^+$ T cells, which likewise fail to produce IL-2 and IFN-γ in response to TCR ligation [90–93].

However, in addition to a reduction in the positive signals leading to cytokine gene promoters, anergized $CD4^+$ T cells suffer from active, dominant repression of the *il2* gene that in some instances can be mapped to distinct sequences in the promoter region [94–96]. Furthermore, repression of *il2* gene expression during anergy induction can behave as a heritable trait that is maintained from a naïve precursor to its daughter cells over several rounds of cell division [73]. These data suggest that gene proximal mechanisms of transcriptional repression are operative in anergic T cells. Indeed, induction of tolerance in both $CD4^+$ and $CD8^+$ T cells is generally associated with increased DNA methylation, histone hypo-acetylation and closed chromatin

structures (see Figure 12.1 and below). Whether T cell tolerance is accompanied by histone modifications that actively contribute to gene silencing (e.g. methylH3K9 and methylH3K27) at cytokine loci is an important question that has not been substantially addressed.

Conventional anergic CD4$^+$ T cells

Induction of histone acetylation, nucleosome remodelling and demethylation of CpG dinucleotides at the *il2* promoter/enhancer in naïve CD4$^+$ T cells requires TCR engagement, but antigenic signals are not sufficient for these changes. Like IL-2 production and anergy avoidance, physicochemical remodelling of the *il2* promoter/enhancer is dependent upon signals from the CD28 co-stimulatory receptor [35]. Naïve CD4$^+$ T cells stimulated in the presence of CTLA4-Ig, which induces anergy by blocking B7–CD28 co-stimulatory interactions, completely fail to remodel and demethylate the *il2* promoter [35]. These defects appear to be a universal feature of CD4$^+$ T cells rendered anergic by distinct tolerogenic signalling modalities (Figure 12.1), as anergy induced *in vitro* with ionomycin or immobilized anti-TCR antibodies (which simulate TCR signalling in the absence of co-stimulation) also exhibit histone hypo-acetylation at the *il2* promoter [97]. These defects are likewise not limited to cells anergized *in vitro*, as superantigen-induced tolerance *in vivo* is associated with histone hypo-acetylation and increased methylation of CpG dinucleotides at the *il2* promoter/enhancer in anergic, superantigen-specific CD4$^+$ T cells [42]. DNA methylation is increased at least 2-fold at each of the four CpG sites within the *il2* promoter/enhancer in anergic compared to effector CD4$^+$ T cells [35,42]. Methylation of each of these sites has been shown to contribute to transcriptional repression of *il2* promoter–reporter constructs [61], and methylation of the −262 CpG located in the ARRE2 element directly blocks Oct1/NFAT binding and negatively regulates transcription of the endogenous *IL2* gene in human CD4$^+$ T cells [66]. Increased methylation at these CpG sites would be expected to contribute to reduced IL-2 production by anergic T cells; however, a clear role for DNA methylation at these sites in the induction of anergy has not been established. Epigenetic modification of the *ifnγ* locus is also affected by tolerogenic signals in CD4$^+$ T cells. Whereas DNA demethylation in this region is only mildly affected in anergic CD4$^+$ T cells [42], histones at the promoter and intronic enhancer are hypo-acetylated compared to effector cells [35,42].

While reduced activity of the CD28-dependent p300/CBP HAT co-activator [57] could contribute to histone hypo-acetylation at the *ifnγ* and *il2* promoters in anergic cells, several lines of evidence suggest that failed histone acetylation is not simply a product of a lack of HAT activity. For instance, histone acetylation is intact at other genes in anergic T cells [35,42,97], indicating that HAT activity is not globally defective. Also, induction of anergy in previously primed T cells, which exhibit increased histone acetylation at the *il2* promoter under resting conditions as compared to naïve or anergic cells [35], results in active histone deacetylation [97], whereas full TCR/CD28 re-stimulation fails to induce histone acetylation at the *il2* promoter in cells previously

rendered anergic [97]. Finally, pharmacological inhibition of HDAC activity during anergy induction restores histone acetylation at the *ifnγ* and *il2* loci in multiple models (see [97] and our unpublished results). These data suggest that HDAC activity maintains nucleosomes in a hypo-acetylated state at cytokine gene loci in anergic T cells. The molecular basis of active histone deacetylation at these genes in anergic cells is becoming clearer, and will be discussed further below.

Tolerant CD8$^+$ T cells

Naïve CD8$^+$ T cells are able to transiently produce IL-2 upon primary stimulation, but as these cells differentiate into effector cytotoxic T lymphocytes, they lose the capacity to produce this growth factor. The inability of effector CD8$^+$ T cells to synthesize IL-2 in response to TCR ligation is associated with reduced MAPK activation [93,98], and has been mapped to a distinct region of the *il2* promoter containing the CD28 response element [99]. CD8$^+$ effector cells also exhibit reduced histone acetylation at this region compared to effector CD4$^+$ cells (Figure 12.1), which are able to produce IL-2 [35,39]. This could result from a lack of c-Rel binding to the CD28RE, which is required for chromatin remodelling at the *il2* promoter [100]. Those CD8$^+$ effector cells that become long-lived memory cells as the immune response progresses regain the capacity to express the *il2* gene; however, this transition is not accompanied by increased histone acetylation or decreased DNA methylation at the *il2* promoter compared to effector cells [39]. The molecular basis for this gain of function is not currently clear, but could result from enhanced TCR-coupled signalling and transcription [98], or differential presence of other epigenetic modifications such as methylation of lysines 4, 9 or 27 of histone H3, for instance.

Induction of stable CD8$^+$ T cell tolerance in several models is also associated with defective TCR-coupled signal transduction [101]; however, epigenetic modifications have only been studied to date in the context of CD8$^+$ T cell tolerance induced by activation in the absence of CD4$^+$ T cell help. CD8$^+$ T cells primed in the absence of CD4$^+$ T cells expand normally, produce IFN-γ and other pro-inflammatory cytokines, gain cytolytic function, and are able to clear pathogen infections during the initial effector phase of the response. However, 'unhelped' CD8$^+$ T cells exhibit defective survival and IFN-γ production compared to helped CD8$^+$ cells during the memory phase, and are unable to protect against subsequent pathogen challenge [57]. Unlike CD8$^+$ T cell anergy induced by co-stimulatory or cytokine blockade, this defective memory phenotype does not appear to result from decreased TCR-proximal signalling, as no defect in MAPK activation could be detected and the anergic phenotype could not be reversed through bypassing proximal TCR-coupled events with phorbol ester and ionomycin [39]. However, anergy in unhelped CD8$^+$ T cells is strongly associated with gene proximal changes in chromatin structure at the *ifnγ* locus. For instance, while virus-specific CD8$^+$ T cells activated *in vivo* by LCMV infection exhibit strong demethylation of CpG dinucleotides within the *ifnγ* promoter and enhancer regardless of CD4$^+$ T cell help, only virus-specific CD8$^+$ T cells that receive CD4$^+$ T cell help

during infection show hyperacetylation of histones in these regions [39]. This defect in histone acetylation at the *ifnγ* locus was already observable at the effector phase, and was maintained for 2 months in the absence of antigenic stimulation (Figure 12.1). Also, while normal $CD8^+$ memory T cells are able to produce IL-2 upon re-stimulation, unhelped $CD8^+$ T cells fail to produce this growth factor, and this defect is associated with increased DNA methylation at the *il2* promoter in the unhelped cells [39].

Foxp3$^+$CD4$^+$ Tregs

Regulatory or suppressor cells are a subset of $CD4^+$ T cells that are anergic; that is, they do not produce cytokines like IL-2 or IFN-γ in response to TCR/CD28 co-stimulation, and in addition can actively inhibit the capacity of neighbouring conventional $CD4^+$ and $CD8^+$ T cells to produce these cytokines [102]. These cells specifically express the forkhead-winged helix transcription factor Foxp3, which is necessary and sufficient for the development and basic functional aspects of this lineage [103–105]. $Foxp3^+CD4^+$ Tregs differentially express thousands of genes as compared to conventional, Foxp3-negative $CD4^+$ cells [104,106,107], and Foxp3 binds directly to a large proportion of these genes [108–111]. While some genes are repressed as a consequence of Foxp3 occupancy, other genes are induced, and the epigenetic modifications present at these genes correlate with their transcriptional activity [109,110]. For instance, binding of Foxp3 to the genes encoding cytotoxic T lymphocyte antigen 4 (CTLA-4) and CD25 induces epigenetic modifications that promote transcription, such as acetylation of histone H3 on Lys-9 and methylation on Lys-4 [109,110]. This is likewise associated with methylation of Lys-27 [110], a modification that has been shown in other systems to promote gene silencing. Conversely, Foxp3 binds to and represses genes such as *pde3b*, *il2* and *ifnγ*, which is accompanied by marked histone deacetylation (Figure 12.1) and Lys-27 methylation at these loci [109,110]. Foxp3 actively recruits HDAC activity to the *il2* and *ifnγ* loci, as Foxp3-mediated deacetylation of these genes is blocked by the HDAC inhibitor trichostatin A (our unpublished results). These epigenetic modifications appear to oppose chromatin remodelling in Treg, as even very strong mitogenic signals are unable to induce DNase sensitivity at the *il2* promoter in these cells [112], and inhibition of HDAC activity in Treg results in increased expression of *ifnγ* and several other Foxp3-responsive genes [113]. This dual function of Foxp3 in directing either histone acetylation or deacetylation in the context of different loci is consistent with the ability of Foxp3 to associate with HATs, HDACs and components of the SWI/SNF chromatin-remodelling complex in cell extracts [114,115]. Interestingly, Foxp3 itself is regulated by acetylation. Inhibiting the interaction between Foxp3 and HATs opposes Foxp3 transcriptional activity [115], and mutation of lysine residues in Foxp3 that are the targets of acetylation abrogates Foxp3 binding to the *il2* promoter, repression of *il2* and suppression of conventional $CD4^+$ T cell responses [113].

In addition, expression of the *foxp3* gene, and therefore Treg development in general, is also regulated epigenetically [116–118]. The *foxp3* locus contains a CpG island that is

fully methylated in conventional Foxp3-negative cells, and almost completely demethylated and acetylated in Foxp3-expressing Treg. Transforming growth factor β (TGF-β), a cytokine that can induce Foxp3 expression in conventional $CD4^+$ T cells, causes demethylation of the *foxp3* CpG island [116]. Experimental inhibition of DNA methylation in non-regulatory cells leads to induction of Foxp3 expression in the absence of TGF-β, while forced methylation of this region in Treg results in loss of Foxp3 [117]. These data suggest that DNA methylation is an important epigenetic mechanism for establishing Treg-specific patterns of gene expression. This mechanism is likely operative at other genes in Treg, as we have observed increased DNA methylation at the *ifnγ* and *il2* promoters in $Foxp3^+$ Treg as compared to conventional $CD4^+$ cells (Figure 12.1 and our unpublished results).

12.5 Targeting epigenetic modifications to cytokine genes in tolerant T cells

As described above, reduced histone acetylation at cytokine genes in multiple types of anergic T cells does not appear to result simply from a failure to recruit HATs, but also involves active maintenance of a deacetylated state through recruitment of HDAC. The enzymes that methylate DNA and modify histones cannot bind DNA by themselves, so how are these factors recruited to cytokine gene loci in anergic T cells? The answer most likely lies in the fact that *de novo* DNA methyltransferases, HDACs and histone methyltransferases exist as components of larger complexes that are recruited by DNA-binding proteins to gene enhancers and promoters as a general mechanism of transcriptional co-repression [119]. While it is clear that Foxp3 is the main factor that targets epigenetic modifications to cytokine genes in regulatory $CD4^+$ T cells, which factor(s) mediate these processes in conventional anergic T cells is not as clear. Several transcriptional repressors capable of recruiting DNA methyltransferase- and/or HDAC-containing complexes are known to bind to the *ifnγ* and *il2* promoters, and recent data have implicated some of these factors in the regulation of T cell anergy. For instance, a number of factors have been identified that negatively regulate *il2* gene expression, including p21SNFT [120], SATB1 [121], ZEB/Nil2a [122], Tob [123], T-bet [124], Egr2 [125,126], Egr3 [126], Smad3 [127,128], CREM [96,129], Ikaros [97,130], NFAT1 [108] and Foxp3 [131]. While most or all of these factors can interact with co-repressor complexes that contain HDACs and/or histone methyltransferases, only some of these factors have been specifically shown to bind to and modulate DNA methylation or chromatin structure at cytokine gene loci, and/or to be involved in the induction of T cell anergy.

The *il2* promoter contains several sequence elements required to oppose the expression of this gene in resting and/or anergic cells. A conserved negative regulatory element (NRE-A) in the *il2* promoter located −105 bp upstream of the transcriptional start site has been shown to bind the zinc-finger transcriptional repressor ZEB/Nil2a [122,132]. This protein is preferentially expressed in resting T cells and especially T cells stimulated in the absence of CD28 co-stimulation, but not in

TCR/CD28-activated Th1 cells [133]. The NRE-A can also be bound by Smad transcription factors. Smad3 is required for repression of *il2* promoter-driven transcription in response to signals from the anti-inflammatory cytokine TGF-β [128], and Smad3 is required for anergy induced by soluble peptide administration *in vivo* [127]. However, this is not a general mechanism operative under all tolerogenic conditions, as Smad3 is unable to repress the *il2* gene in the absence of TGF-β [128]. Egr2 and Egr3 are transcription factors that are preferentially induced under anergic conditions, and these factors are required for full tolerance induction in multiple models [125,126]. However, while the positive regulator Egr1 can bind the *il2* promoter, neither Egr2 nor Egr3 have been shown to bind directly to the *il2* or *ifn*γ loci. Egr2, Egr3, Smad3 and ZEB/Nil2a are each known to interact with HDAC-containing co-repressor complexes; however, a role for these factors in the regulation of chromatin structure or DNA methylation at the *il2* or other cytokine genes has not been established.

The distal AP-1 site at −180 bp of the *il2* promoter/enhancer has been shown to bind CREB [56]. This factor normally binds to DNA as a CREB/CREB homodimer that can recruit the p300/CBP HAT via the CREB transactivation domain [134]. However, CREB can also bind to a splice variant termed CREM, which lacks the transactivation domain, rendering CREB/CREM heterodimers unable to recruit HAT co-activators [135]. Inhibition of CREM expression in activated $CD4^+$ T cells leads to increased histone acetylation at the *il2* promoter and enhanced IL-2 production [129]. In anergic $CD4^+$ T cell clones CREB/CREM heterodimers are preferentially bound to the −180 bp AP-1 site, and mutation of this site leads to reduced CREM binding and resistance to anergy induction [96]. The contrast of binding of CREB as a homodimer or heterodimer with CREM could therefore act as a biochemical switch that turns local histone acetylation on or off through the differential capacity to recruit HATs. Another such biochemical switch that could operate at the *ifn*γ and *il2* loci involves NF-κB, the activity of which is controlled both through regulated cytoplasmic sequestration by inhibitor of NF-κB α (IκBα), and by phosphorylation [136]. Once targeted to the nucleus, NF-κB is not able to transactivate by itself, but requires HAT co-activators to promote gene transcription [137]. The ability of NF-κB to recruit co-activators is regulated by its phosphorylation state. If non-phosphorylated, NF-κB is bound to DNA, fails to recruit HAT co-activators and binds to HDACs and acts as a transcriptional repressor [138]. A role for this switch in regulating chromatin structure at cytokine genes has not been specifically established, but consistent with this scenario p50/p50 homodimers are known to bind to the IL2kB element within the TCE_d/NFIL-2C region of the *il2* promoter, and this activity is required for full repression of *il2* gene expression in quiescent and anergic T cells [139,140].

A factor that has recently been shown to play a crucial role in the epigenetic regulation of cytokine genes and anergy in T cells is Ikaros. This zinc-finger DNA-binding protein was originally identified as a factor required for lymphoid development, where it regulates antigen-receptor genes and genes involved in VDJ recombination by recruiting chromatin-remodelling complexes such as NURD, CtBP and Sin3 [6]. Ikaros is highly expressed in mature, naïve $CD4^+$ T cells, where it binds to specific elements in the *il2* promoter [130] and the *ifn*γ promoter and enhancer regions (our unpublished results).

Ikaros–*il2* promoter complexes derived from $CD4^+$ T cells are enriched for HDAC1, HDAC2 and hypo-acetylated histones H3 and H4 [97,130]. Ikaros occupancy is responsible for the hypo-acetylated state of the histones at the *il2* promoter in quiescent cells, as naïve $CD4^+$ T cells from mice with defective Ikaros DNA-binding activity exhibit constitutive histone hyperacetylation at the *il2* promoter [130]. Thus, in the absence of Ikaros function, the *il2* gene resembles a transcriptionally 'poised' locus, and consistent with this, Ikaros-defective naïve $CD4^+$ T cells show enhanced expression of the *il2* gene upon optimal TCR/CD28 co-stimulation [130]. Conversely, overexpression of Ikaros in effector $CD4^+$ T cells reduces the capacity of these cells to IL-2 production upon TCR/CD28 stimulation [97,130]. Also, while normal naïve T cells require CD28 co-stimulation to acheive histone acetylation and transcription from the *il2* promoter, naïve $CD4^+$ T cells from Ikaros-deficient mice can produce wild-type levels of IL-2 and IFN-γ in response to TCR engagement alone ([130] and our unpublished results). Consequently, $CD4^+$ T cells with defective Ikaros DNA-binding activity are highly resistant to anergy induced by CD28 or IL-2 blockade [130], or by calcium ionophore treatment [97].

How is DNA methylation maintained at cytokine loci during anergy induction? Much less information is available regarding molecular mechanisms of DNA methylation in tolerant cells, and one reason for this is that the means by which demethylation is achieved in productively stimulated effector or memory T cells is not understood. The bond between the methyl group and the cytosine sugar is considered too strong to allow enzymic cleavage; therefore, the standard model has posited that DNA is demethylated passively through the failure of DNA methyltransferase 1 to methylate CpG dinucleotides in the new daughter strand during DNA replication in proliferating cells. In this scenario, the failure to demethylate cytokine loci in anergic T cells would be coupled to reduced DNA synthesis, as many modes of tolerance induction are associated with abortive T cell-cycle progression [141,142]. However, recent studies have shown that CpG methylation at the *il2* promoter in naive $CD4^+$ T cells [61,66], the *il4* locus in Th2 cells [143], and the *ifn*γ promoter in memory $CD8^+$ T cells [43] is rapidly lost in response to antigenic stimuli in a replication-independent manner. The mechanism by which this process occurs was not determined in these studies; however, a solution to this paradox may lie in two recent papers showing that the promoters of oestrogen-responsive genes can oscillate rapidly between methylated and demethylated states during transcription, as a result of cyclical recruitment of *de novo* DNA methyltransferases that also mediate DNA deamidation. Importantly, these studies show that demethylation of DNA in mammalian cells does not require breakage of the methylcytosine C–C bond, but can result instead from excision of the methylated cytosine and replacement with a non-methylated residue. If this dynamic also operates at cytokine promoters in T cells responding to antigenic stimuli, then the DNA demethylation observed over time in productively activated cells would result from a shift in the overall balance from methyltransferase to deamidase activity. Conversely, the lack of demethylation (or increased DNA methylation) observed at cytokine loci in anergic T cells would be attributed to a shift in the overall balance from deamidase to methyltransferase

activity. For instance, transcriptional repressors that bind the *il2* and/or *ifnγ* genes in anergic cells (e.g. NF-κB p50, CREM, Ikaros) may promote recruitment of DNA methyltransferase 3a, as has been shown to occur at the epigenetically silenced *il4* locus in differentiating Th1 cells [144]. Alternatively, deamidase and/or T → C DNA repair activity may selectively depend upon co-stimulatory signals. Although these new data represent significant progress in our understanding of how this epigenetic process is regulated in T cells, further research will be required to test these ideas.

12.6 Common mechanisms of epigenetic silencing among distinct types of tolerant T cells?

The epigenetic modifications present at silenced cytokine genes in regulatory $CD4^+$ T cells, conventional anergic $CD4^+$ T cells and tolerant $CD8^+$ T cells are remarkably similar; that is, increased DNA methylation, decreased histone acetylation and decreased accessibility of the chromatin to DNA-binding proteins. These modifications are likewise reminiscent of those present at the silenced *ifnγ* and *il4* genes in polarized Th1 and Th2 cells [7–9]. Do the same mechanisms operate to silence cytokine genes in all tolerant and polarized T cells? Many of these cell types express lineage-specific transcription factors that are responsible for establishing the epigenetic patterns that are unique to that cell type. For example, Foxp3 is specifically expressed by $CD4^+CD25^+$ Tregs, and is directly responsible for recruiting HAT or HDAC activity to Foxp3-responsive genes. Similarly, T-bet, which is exclusively expressed by polarized Th1 $CD4^+$ T cells, and GATA-3, a Th2-specific factor, are each sufficient to induce DNA demethylation and histone acetylation at the *ifnγ* and *il4* genes, respectively [9]. By definition, these factors represent unique mechanisms of coupling DNA- and chromatin-modifying enzymes to gene promoters and enhancers that cannot be utilized by all cell types. However, it is possible that these lineage-specific factors must cooperate with general DNA-binding proteins that are common to all the subsets (e.g. Ikaros) in order to epigenetically silence cytokine genes upon the induction of tolerance. In this way, similar epigenetic machinery could be targeted to specific genes in different cell types. A clearer understanding of the mechanisms by which cytokine genes are silenced in T cells must await further research.

References

1. Hashimshony, T., Zhang, J., Keshet, I. *et al.* (2003) The role of DNA methylation in setting up chromatin structure during development. *Nature Genetics* **34**, 187–192.
2. Gaudet, F., Hodgson, J.G., Eden, A. *et al.* (2003) Induction of tumors in mice by genomic hypomethylation. *Science* **300**, 489–492.
3. Eden, A., Gaudet, F., Waghmare, A. *et al.* (2003) Chromosomal instability and tumors promoted by DNA hypomethylation. *Science* **300**, 455.

4. Smale, S.T. and Fisher, A.G. (2002) Chromatin structure and gene regulation in the immune system. *Annual Review of Immunology* **20**, 427–462.
5. Wilson, C.B., Makar, K.W. and Perez-Melgosa, M. (2002) Epigenetic regulation of T cell fate and function. *Journal of Infectious Diseases* **185**(suppl. 1), S37–S45.
6. Georgopoulos, K. (2002) Haematopoietic cell-fate decisions, chromatin regulation and ikaros. *Nature Reviews Immunology* **2**, 162–174.
7. Wilson, C.B. and Merkenschlager, M. (2006) Chromatin structure and gene regulation in T cell development and function. *Current Opinion in Immunology* **18**, 143–151.
8. Ansel, K.M., Lee, D.U. and Rao, A. (2003) An epigenetic view of helper T cell differentiation. *Nature Immunology* **4**, 616–623.
9. Lee, G.R., Kim, S.T., Spilianakis, C.G. *et al.* (2006) T helper cell differentiation: regulation by cis elements and epigenetics. *Immunity* **24**, 369–379.
10. Workman, J.L. and Kingston, R.E. (1998) Alteration of nucleosome structure as a mechanism of transcriptional regulation. *Annual Review of Biochemistry* **67**, 545–579.
11. Jenuwein, T. and Allis, C.D. (2001) Translating the histone code. *Science* **293**, 1074–1080.
12. Berger, S.L. (2002) Histone modifications in transcriptional regulation. *Current Opinion in Genetics and Development* **12**, 142–148.
13. Narlikar, G.J., Fan, H.Y. and Kingston, R.E. (2002) Cooperation between complexes that regulate chromatin structure and transcription. *Cell* **108**, 475–487.
14. Roth, S.Y., Denu, J.M. and Allis, C.D. (2001) Histone acetyltransferases. *Annual Review of Biochemistry* **70**, 81–120.
15. Berger, S.L. (1999) Gene activation by histone and factor acetyltransferases. *Current Opinion in Cell Biology* **11**, 336–341.
16. Fuks, F., Burgers, W.A., Brehm, A. *et al.* (2000) DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nature Genetics* **24**, 88–91.
17. Fuks, F., Hurd, P.J., Deplus, R. *et al.* (2003) The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase. *Nucleic Acids Research* **31**, 2305–2312.
18. Fuks, F., Hurd, P.J., Wolf, D. *et al.* (2003) The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *Journal of Biological Chemistry* **278**, 4035–4040.
19. Lomvardas, S. and Thanos, D. (2002) Modifying gene expression programs by altering core promoter chromatin architecture. *Cell* **110**, 261–271.
20. Roebuck, K.A. (1999) Regulation of interleukin-8 gene expression. *Journal of Interferon and Cytokine Research* **19**, 429–438.
21. Lomvardas, S. and Thanos, D. (2001) Nucleosome sliding via TBP DNA binding in vivo. *Cell* **106**, 685–696.
22. Maniatis, T., Falvo, J.V., Kim, T.H. *et al.* (1998) Structure and function of the interferon-beta enhanceosome. *Cold Spring Harbor Symposia on Quantitative Biology* **63**, 609–620.
23. Kim, T.K. and Maniatis, T. (1997) The mechanism of transcriptional synergy of an in vitro assembled interferon-beta enhanceosome. *Molecular Cell* **1**, 119–129.
24. Jain, J., Loh, C. and Rao, A. (1995) Transcriptional regulation of the IL-2 gene. *Current Opinion in Immunology* **7**, 333–342.
25. Serfling, E., Avots, A. and Neumann, M. (1995) The architecture of the interleukin-2 promoter: a reflection of T lymphocyte activation. *Biochimica et Biophysica Acta* **1263**, 181–200.
26. Bunting, K., Wang, J. and Shannon, M.F. (2006) Control of interleukin-2 gene transcription: a paradigm for inducible, tissue-specific gene expression. *Vitamins and Hormones* **74**, 105–145.
27. Schoenborn, J.R. and Wilson, C.B. (2007) Regulation of interferon-gamma during innate and adaptive immune responses. *Advances in Immunology* **96**, 41–101.
28. Yui, M.A., Hernandez-Hoyos, G. and Rothenberg, E.V. (2001) A new regulatory region of the IL-2 locus that confers position-independent transgene expression. *Journal of Immunology* **166**, 1730–1739.

29. Peters, A.H. and Schubeler, D. (2005) Methylation of histones: playing memory with DNA. *Current Opinion in Cell Biology* **17**, 230–238.
30. Adachi, S. and Rothenberg, E.V. (2005) Cell-type-specific epigenetic marking of the IL2 gene at a distal cis-regulatory region in competent, nontranscribing T-cells. *Nucleic Acids Research* **33**, 3200–3210.
31. Siebenlist, U., Durand, D.B., Bressler, P. *et al.* (1986) Promoter region of interleukin-2 gene undergoes chromatin structure changes and confers inducibility on chloramphenicol acetyltransferase gene during activation of T cells. *Molecular and Cellular Biology* **6**, 3042–3049.
32. Ward, S.B., Hernandez-Hoyos, G., Chen, F. *et al.* (1998) Chromatin remodeling of the interleukin-2 gene: distinct alterations in the proximal versus distal enhancer regions. *Nucleic Acids Research* **26**, 2923–2934.
33. Rao, S., Procko, E. and Shannon, M.F. (2001) Chromatin remodeling, measured by a novel real-time polymerase chain reaction assay, across the proximal promoter region of the IL-2 gene. *Journal of Immunology* **167**, 4494–4503.
34. Attema, J.L., Reeves, R., Murray, V. *et al.* (2002) The human IL-2 gene promoter can assemble a positioned nucleosome that becomes remodeled upon T cell activation. *Journal of Immunology* **169**, 2466–2476.
35. Thomas, R.M., Gao, L. and Wells, A.D. (2005) Signals from CD28 induce stable epigenetic modification of the IL-2 promoter. *Journal of Immunology* **174**, 4639–4646.
36. Agarwal, S. and Rao, A. (1998) Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. *Immunity* **9**, 765–775.
37. Fields, P.E., Kim, S.T. and Flavell, R.A. (2002) Cutting edge: changes in histone acetylation at the IL-4 and IFN-gamma loci accompany Th1/Th2 differentiation. *Journal of Immunology* **169**, 647–650.
38. Avni, O., Lee, D., Macian, F. *et al.* (2002) T(H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. *Nature Immunology* **3**, 643–651.
39. Northrop, J.K., Thomas, R.M., Wells, A.D. *et al.* (2006) Epigenetic remodeling of the IL-2 and IFN-gamma loci in memory CD8 T cells is influenced by CD4 T cells. *Journal of Immunology* **177**, 1062–1069.
40. Shannon, M.F., Himes, S.R. and Attema, J. (1998) A role for the architectural transcription factors HMGI(Y) in cytokine gene transcription in T cells. *Immunology and Cell Biology* **76**, 461–466.
41. Himes, S.R., Reeves, R., Attema, J. *et al.* (2000) The role of high-mobility group I(Y) proteins in expression of IL-2 and T cell proliferation. *Journal of Immunology* **164**, 3157–3168.
42. Thomas, R.M., Saouaf, S.J. and Wells, A.D. (2007) Superantigen-induced CD4+ T cell tolerance is associated with DNA methylation and histone hypo-acetylation at cytokine gene loci. *Genes and Immunity* **8**, 613–618.
43. Kersh, E.N., Fitzpatrick, D.R., Murali-Krishna, K. *et al.* (2006) Rapid demethylation of the IFN-gamma gene occurs in memory but not naive CD8 T cells. *Journal of Immunology* **176**, 4083–4093.
44. Winders, B.R., Schwartz, R.H. and Bruniquel, D. (2004) A distinct region of the murine IFN-gamma promoter is hypomethylated from early T cell development through mature naive and Th1 cell differentiation, but is hypermethylated in Th2 cells. *Journal of Immunology* **173**, 7377–7384.
45. Bird, J.J., Brown, D.R., Mullen, A.C. *et al.* (1998) Helper T cell differentiation is controlled by the cell cycle. *Immunity* **9**, 229–237.
46. Fitzpatrick, D.R., Shirley, K.M. and Kelso, A. (1999) Cutting edge: stable epigenetic inheritance of regional IFN-gamma promoter demethylation in CD44highCD8+ T lymphocytes. *Journal of Immunology* **162**, 5053–5057.

47. Santangelo, S., Cousins, D.J., Winkelmann, N.E. *et al.* (2002) DNA methylation changes at human Th2 cytokine genes coincide with DNase I hypersensitive site formation during CD4(+) T cell differentiation. *Journal of Immunology* **169**, 1893–1903.
48. Lee, D.U., Agarwal, S. and Rao, A. (2002) Th2 lineage commitment and efficient IL-4 production involves extended demethylation of the IL-4 gene. *Immunity* **16**, 649–660.
49. Lee, D.U., Avni, O., Chen, L. *et al.* (2003) A distal enhancer in the IFN-gamma locus revealed by genome sequence comparison. *Journal of Biological Chemistry* **279**, 4802–4810.
50. Shnyreva, M., Weaver, W.M., Blanchette, M. *et al.* (2004) Evolutionarily conserved sequence elements that positively regulate IFN-gamma expression in T cells. *Proceedings of the National Academy of Sciences USA* **101**, 12622–12627.
51. Rothenberg, E.V. and Ward, S.B. (1996) A dynamic assembly of diverse transcription factors integrates activation and cell-type information for interleukin 2 gene regulation. *Proceedings of the National Academy of Sciences USA* **93**, 9358–9365.
52. Sica, A., Dorman, L., Viggiano, V. *et al.* (1997) Interaction of NF-kappaB and NFAT with the interferon-gamma promoter. *Journal of Biological Chemistry* **272**, 30412–30420.
53. Avots, A., Buttmann, M., Chuvpilo, S. *et al.* (1999) CBP/p300 integrates Raf/Rac-signaling pathways in the transcriptional induction of NF-ATc during T cell activation. *Immunity* **10**, 515–524.
54. Garcia-Rodriguez, C. and Rao, A. (1998) Nuclear factor of activated T cells (NFAT)-dependent transactivation regulated by the coactivators p300/CREB-binding protein (CBP). *Journal of Experimental Medicine* **187**, 2031–2036.
55. Shapiro, V.S., Truitt, K.E., Imboden, J.B. *et al.* (1997) CD28 mediates transcriptional upregulation of the interleukin-2 (IL-2) promoter through a composite element containing the CD28RE and NF-IL-2B AP-1 sites. *Molecular and Cellular Biology* **17**, 4051–4058.
56. Butscher, W.G., Haggerty, C.M., Chaudhry, S. *et al.* (2001) Targeting of p300 to the interleukin-2 promoter via CREB-Rel cross-talk during mitogen and oncogenic molecular signaling in activated T-cells. *Journal of Biological Chemistry* **276**, 27647–27656.
57. Nandiwada, S.L., Li, W., Zhang, R. *et al.* (2006) p300/Cyclic AMP-responsive element binding-binding protein mediates transcriptional coactivation by the CD28 T cell costimulatory receptor. *Journal of Immunology* **177**, 401–413.
58. Zhao, K., Wang, W., Rando, O.J. *et al.* (1998) Rapid and phosphoinositol-dependent binding of the SWI/SNF-like BAF complex to chromatin after T lymphocyte receptor signaling. *Cell* **95**, 625–636.
59. Spilianakis, C.G. and Flavell, R.A. (2004) Long-range intrachromosomal interactions in the T helper type 2 cytokine locus. *Nature Immunology* **5**, 1017–1027.
60. Eivazova, E.R. and Aune, T.M. (2004) Dynamic alterations in the conformation of the Ifng gene region during T helper cell differentiation. *Proceedings of the National Academy of Sciences USA* **101**, 251–256.
61. Bruniquel, D. and Schwartz, R.H. (2003) Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. *Nature Immunology* **4**, 235–240.
62. Ballas, Z.K. (1984) The use of 5-azacytidine to establish constitutive interleukin 2-producing clones of the EL4 thymoma. *Journal of Immunology* **133**, 7–9.
63. Lee, P.P., Fitzpatrick, D.R., Beard, C. *et al.* (2001) A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity* **15**, 763–774.
64. Makar, K.W. and Wilson, C.B. (2004) DNA methylation is a nonredundant repressor of the Th2 effector program. *Journal of Immunology* **173**, 4402–4406.
65. Penix, L., Weaver, W.M., Pang, Y. *et al.* (1993) Two essential regulatory elements in the human interferon gamma promoter confer activation specific expression in T cells. *Journal of Experimental Medicine* **178**, 1483–1496.

66. Murayama, A., Sakura, K., Nakama, M. *et al.* (2006) A specific CpG site demethylation in the human interleukin 2 gene promoter is an epigenetic memory. *EMBO Journal* **25**, 1081–1092.
67. Mullen, A.C., Hutchins, A.S., Villarino, A.V. *et al.* (2001) Cell cycle controlling the silencing and functioning of mammalian activators. *Current Biology* **11**, 1695–1699.
68. Hutchins, A.S., Mullen, A.C., Lee, H.W. *et al.* (2002) Gene silencing quantitatively controls the function of a developmental trans-activator. *Molecular Cell* **10**, 81–91.
69. Hollander, G.A., Zuklys, S., Morel, C. *et al.* (1998) Monoallelic expression of the interleukin-2 locus. *Science* **279**, 2118–2121.
70. Chiodetti, L., Barber, D.L. and Schwartz, R.H. (2000) Biallelic expression of the IL-2 locus under optimal stimulation conditions. *European Journal of Immunology* **30**, 2157–2163.
71. Gett, A.V. and Hodgkin, P.D. (1998) Cell division regulates the T cell cytokine repertoire, revealing a mechanism underlying immune class regulation. *Proceedings of the National Academy of Sciences USA* **95**, 9488–9493.
72. Gudmundsdottir, H., Wells, A.D. and Turka, L.A. (1999) Dynamics and requirements of T cell clonal expansion in vivo at the single-cell level: effector function is linked to proliferative capacity. *Journal of Immunology* **162**, 5212–5223.
73. Wells, A.D., Walsh, M.C., Bluestone, J.A. *et al.* (2001) Signaling through CD28 and CTLA-4 controls two distinct forms of T cell anergy. *Journal of Clinical Investigation* **108**, 895–903.
74. Wells, A.D., Walsh, M.C., Sankaran, D. *et al.* (2000) T cell effector function and anergy avoidance are quantitatively linked to cell division. *Journal of Immunology* **165**, 2432–2443.
75. Schwartz, R.H. (2003) T cell anergy. *Annual Review of Immunology* **21**, 305–334.
76. Harding, F.A., McArthur, J.G., Gross, J.A. *et al.* (1992) CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature* **356**, 607–609.
77. Mescher, M.F., Popescu, F.E., Gerner, M. *et al.* (2007) Activation-induced non-responsiveness (anergy) limits CD8 T cell responses to tumors. *Seminars in Cancer Biology* **17**, 299–308.
78. Redmond, W.L. and Sherman, L.A. (2005) Peripheral tolerance of CD8 T lymphocytes. *Immunity* **22**, 275–284.
79. Bourgeois, C. and Tanchot, C. (2003) Mini-review CD4 T cells are required for CD8 T cell memory generation. *European Journal of Immunology* **33**, 3225–3231.
80. Shedlock, D.J. and Shen, H. (2003) Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* **300**, 337–339.
81. Mueller, D.L. (2004) E3 ubiquitin ligases as T cell anergy factors. *Nature Immunology* **5**, 883–890.
82. Wells, A.D., Liu, Q.H., Hondowicz, B. *et al.* (2003) Regulation of T cell activation and tolerance by phospholipase Cgamma-1-dependent integrin avidity modulation. *Journal of Immunology* **170**, 4127–4133.
83. Boussiotis, V.A., Barber, D.L., Lee, B.J. *et al.* (1996) Differential association of protein tyrosine kinases with the T cell receptor is linked to the induction of anergy and its prevention by B7 family-mediated costimulation. *Journal of Experimental Medicine* **184**, 365–376.
84. Hundt, M., Tabata, H., Jeon, M.S. *et al.* (2006) Impaired activation and localization of LAT in anergic T cells as a consequence of a selective palmitoylation defect. *Immunity* **24**, 513–522.
85. DeSilva, D.R., Feeser, W.S., Tancula, E.J. *et al.* (1996) Anergic T cells are defective in both jun NH2-terminal kinase and mitogen-activated protein kinase signaling pathways. *Journal of Experimental Medicine* **183**, 2017–2023.
86. Fields, P.E., Gajewski, T.F. and Fitch, F.W. (1996) Blocked Ras activation in anergic CD4 + T cells. *Science* **271**, 1276–1278.
87. Li, W., Whaley, C.D., Mondino, A. *et al.* (1996) Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4 + T cells. *Science* **271**, 1272–1276.

88. Sloan-Lancaster, J., Steinberg, T.H. and Allen, P.M. (1996) Selective activation of the calcium signaling pathway by altered peptide ligands. *Journal of Experimental Medicine* **184**, 1525–1530.
89. Boussiotis, V.A., Freeman, G.J., Berezovskaya, A. *et al.* (1997) Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1. *Science* **278**, 124–128.
90. Li, L., Godfrey, W.R., Porter, S.B. *et al.* (2005) CD4 + CD25 + regulatory T-cell lines from human cord blood have functional and molecular properties of T-cell anergy. *Blood* **106**, 3068–3073.
91. Hickman, S.P., Yang, J., Thomas, R.M. *et al.* (2006) Defective activation of protein kinase C and Ras-ERK pathways limits IL-2 production and proliferation by CD4 + CD25 + regulatory T cells. *Journal of Immunology* **177**, 2186–2194.
92. Crellin, N.K., Garcia, R.V. and Levings, M.K. (2007) Altered activation of AKT is required for the suppressive function of human CD4 + CD25 + T regulatory cells. *Blood* **109**, 2014–2022.
93. Tham, E.L. and Mescher, M.F. (2001) Signaling alterations in activation-induced nonresponsive CD8 T cells. *Journal of Immunology* **167**, 2040–2048.
94. Telander, D.G., Malvey, E.N. and Mueller, D.L. (1999) Evidence for repression of IL-2 gene activation in anergic T cells. *Journal of Immunology* **162**, 1460–1465.
95. Kitagawa-Sakakida, S. and Schwartz, R.H. (1996) Multifactor cis-dominant negative regulation of IL-2 gene expression in anergized T cells. *Journal of Immunology* **157**, 2328–2339.
96. Powell, J.D., Lerner, C.G., Ewoldt, G.R. *et al.* (1999) The -180 site of the IL-2 promoter is the target of CREB/CREM binding in T cell anergy. *Journal of Immunology* **163**, 6631–6639.
97. Bandyopadhyay, S., Dure, M., Paroder, M. *et al.* (2006) Interleukin 2 gene transcription is regulated by Ikaros-induced changes in histone acetylation in anergic T cells. *Blood* **109**, 2878–2886.
98. Kaech, S.M., Hemby, S., Kersh, E. *et al.* (2002) Molecular and functional profiling of memory CD8 T cell differentiation. *Cell* **111**, 837–851.
99. Finch, R.J., Fields, P.E. and Greenberg, P.D. (2001) A transcriptional block in the IL-2 promoter at the -150 AP-1 site in effector CD8 + T cells. *Journal of Immunology* **166**, 6530–6536.
100. Rao, S., Gerondakis, S., Woltring, D. *et al.* (2003) c-Rel is required for chromatin remodeling across the IL-2 gene promoter. *Journal of Immunology* **170**, 3724–3731.
101. Mescher, M.F., Agarwal, P., Casey, K.A. *et al.* (2007) Molecular basis for checkpoints in the CD8 T cell response: tolerance versus activation. *Seminars in Immunology* **19**, 153–161.
102. Sakaguchi, S. (2004) Naturally arising CD4 + regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annual Review of Immunology* **22**, 531–562.
103. Khattri, R., Cox, T., Yasayko, S.A. *et al.* (2003) An essential role for Scurfin in CD4 + CD25 + T regulatory cells. *Nature Immunology* **4**, 337–342.
104. Fontenot, J.D., Rasmussen, J.P., Williams, L.M. *et al.* (2005) Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* **22**, 329–341.
105. Hori, S., Nomura, T. and Sakaguchi, S. (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057–1061.
106. Sugimoto, N., Oida, T., Hirota, K. *et al.* (2006) Foxp3-dependent and -independent molecules specific for CD25 + CD4 + natural regulatory T cells revealed by DNA microarray analysis. *International Immunology* **18**, 1197–1209.
107. Gavin, M.A., Rasmussen, J.P., Fontenot, J.D. *et al.* (2007) Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* **445**, 771–775.
108. Wu, Y., Borde, M., Heissmeyer, V. *et al.* (2006) FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* **126**, 375–387.
109. Chen, C., Rowell, E.A., Thomas, R.M. *et al.* (2006) Transcriptional regulation by Foxp3 is associated with direct promoter occupancy and modulation of histone acetylation. *Journal of Biological Chemistry* **281**, 36828–36834.

110. Zheng, Y., Josefowicz, S.Z., Kas, A. *et al.* (2007) Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature* **445**, 936–940.
111. Marson, A., Kretschmer, K., Frampton, G.M. *et al.* (2007) Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature* **445**, 931–935.
112. Su, L., Creusot, R.J., Gallo, E.M. *et al.* (2004) Murine CD4 + CD25 + regulatory T cells fail to undergo chromatin remodeling across the proximal promoter region of the IL-2 gene. *Journal of Immunology* **173**, 4994–5001.
113. Tao, R., de Zoeten, E.F., Ozkaynak, E. *et al.* (2007) Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nature Medicine* **13**, 1299–1307.
114. Grant, C., Oh, U., Fugo, K. *et al.* (2006) Foxp3 represses retroviral transcription by targeting both NF-kappaB and CREB pathways. *PLoS Pathogens* **2**, e33.
115. Li, B., Samanta, A., Song, X. *et al.* (2007) FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. *Proceedings of the National Academy of Sciences USA* **104**, 4571–4576.
116. Floess, S., Freyer, J., Siewert, C. *et al.* (2007) Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biology* **5**, e38.
117. Kim, H.P. and Leonard, W.J. (2007) CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. *Journal of Experimental Medicine* **204**, 1543–1551.
118. Baron, U., Floess, S., Wieczorek, G. *et al.* (2007) DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. *European Journal of Immunology* **37**, 2378–2389.
119. Ng, H.H. and Bird, A. (2000) Histone deacetylases: silencers for hire. *Trends in Biochemical Sciences* **25**, 121–126.
120. Iacobelli, M., Wachsman, W. and McGuire, K.L. (2000) Repression of IL-2 promoter activity by the novel basic leucine zipper p21SNFT protein. *Journal of Immunology* **165**, 860–868.
121. Kumar, P.P., Purbey, P.K., Ravi, D.S. *et al.* (2005) Displacement of SATB1-bound histone deacetylase 1 corepressor by the human immunodeficiency virus type 1 transactivator induces expression of interleukin-2 and its receptor in T cells. *Molecular and Cellular Biology* **25**, 1620–1633.
122. Williams, T.M., Moolten, D., Burlein, J. *et al.* (1991) Identification of a zinc finger protein that inhibits IL-2 gene expression. *Science* **254**, 1791–1794.
123. Tzachanis, D., Freeman, G.J., Hirano, N. *et al.* (2001) Tob is a negative regulator of activation that is expressed in anergic and quiescent T cells. *Nature Immunology*, **2**, 1174–1182.
124. Szabo, S.J., Kim, S.T., Costa, G.L. *et al.* (2000) A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* **100**, 655–669.
125. Harris, J.E., Bishop, K.D., Phillips, N.E. *et al.* (2004) Early growth response gene-2, a zinc-finger transcription factor, is required for full induction of clonal anergy in CD4 + T cells. *Journal of Immunology* **173**, 7331–7338.
126. Safford, M., Collins, S., Lutz, M.A. *et al.* (2005) Egr-2 and Egr-3 are negative regulators of T cell activation. *Nature Immunology* **6**, 472–480.
127. Li, L., Iwamoto, Y., Berezovskaya, A. *et al.* (2006) A pathway regulated by cell cycle inhibitor p27 (Kip1) and checkpoint inhibitor Smad3 is involved in the induction of T cell tolerance. *Nature Immunology* **7**, 1157–1165.
128. McKarns, S.C., Schwartz, R.H. and Kaminski, N.E. (2004) Smad3 is essential for TGF-beta 1 to suppress IL-2 production and TCR-induced proliferation, but not IL-2-induced proliferation. *Journal of Immunology* **172**, 4275–4284.
129. Tenbrock, K., Juang, Y.T., Tolnay, M. *et al.* (2003) The cyclic adenosine 5′-monophosphate response element modulator suppresses IL-2 production in stimulated T cells by a chromatin-dependent mechanism. *Journal of Immunology* **170**, 2971–2976.

130. Thomas, R.M., Chunder, N., Chen, C. *et al.* (2007) Ikaros enforces the costimulatory requirement for IL2 gene expression and is required for anergy induction in CD4+ T lymphocytes. *Journal of Immunology* **179**, 7305–7315.
131. Schubert, L.A., Jeffery, E., Zhang, Y. *et al.* (2001) Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. *Journal of Biological Chemistry* **276**, 37672–37679.
132. Yasui, D.H., Genetta, T., Kadesch, T. *et al.* (1998) Transcriptional repression of the IL-2 gene in Th cells by ZEB. *Journal of Immunology* **160**, 4433–4440.
133. Becker, J.C., Brabletz, T., Kirchner, T. *et al.* (1995) Negative transcriptional regulation in anergic T cells. *Proceedings of the National Academy of Sciences USA* **92**, 2375–2378.
134. Eckner, R. (1996) p300 and CBP as transcriptional regulators and targets of oncogenic events. *Biological Chemistry* **377**, 685–688.
135. Brindle, P., Linke, S. and Montminy, M. (1993) Protein-kinase-A-dependent activator in transcription factor CREB reveals new role for CREM repressors. *Nature* **364**, 821–824.
136. Karin, M. and Ben-Neriah, Y. (2000) Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annual Review of Immunology* **18**, 621–663.
137. Sheppard, K.A., Rose, D.W., Haque, Z.K. *et al.* (1999) Transcriptional activation by NF-kappaB requires multiple coactivators. *Molecular and Cellular Biology* **19**, 6367–6378.
138. Zhong, H., May, M.J., Jimi, E. *et al.* (2002) The phosphorylation status of nuclear NF-kappa B determines its association with CBP/p300 or HDAC-1. *Molecular Cell* **9**, 625–636.
139. Grundstrom, S., Anderson, P., Scheipers, P. *et al.* (2004) Bcl-3 and NFkappaB p50-p50 homodimers act as transcriptional repressors in tolerant CD4+ T cells. *Journal of Biological Chemistry* **279**, 8460–8468.
140. Kang, S.M., Tran, A.C., Grilli, M. *et al.* (1992) NF-kappa B subunit regulation in nontransformed CD4+ T lymphocytes. *Science* **256**, 1452–1456.
141. Wells, A.D. (2003) Cell-cycle regulation of T-cell responses–novel approaches to the control of alloimmunity. *Immunological Reviews* **196**, 25–36.
142. Wells, A.D. (2007) Cyclin-dependent kinases: molecular switches controlling anergy and potential therapeutic targets for tolerance. *Seminars in Immunology* **19**, 173–179.
143. Kim, S.T., Fields, P.E. and Flavell, R.A. (2007) Demethylation of a specific hypersensitive site in the Th2 locus control region. *Proceedings of the National Academy of Sciences USA* **104**, 17052–17057.
144. Makar, K.W., Perez-Melgosa, M., Shnyreva, M. *et al.* (2003) Active recruitment of DNA methyltransferases regulates interleukin 4 in thymocytes and T cells. *Nature Immunology* **4**, 1183–1190.

Abstract

The DNA that constitutes each chromosome in a eukaryotic cell is wrapped around protein complexes called nucleosomes, which are in turn incorporated into higher-order structures called chromatin, allowing the efficient packaging of the approximately 3 billion nucleotides of our genome into each cell's nucleus. In addition, mammalian DNA can be methylated at cytosine residues. Whether a gene is methylated, is wrapped tightly around a nucleosome and whether the nucleosomes are in turn packed together loosely or tightly into chromatin, can influence its expression. Moreover, these physicochemical aspects of a gene's structure can be maintained throughout DNA replication and inherited by the daughter cells after cell division. Such 'epigenetic' mechanisms of gene regulation are known to play crucial roles in developmental biology [1] and cancer [2,3], and their involvement in regulating fate decisions by cells of the immune system is becoming appreciated [4–9]. Here we describe the types of epigenetic modifications that occur at T cell effector cytokine genes during an immune response, and how the physical structure of these loci is affected by signals that induce acquired immune tolerance.

Key words: epigenetics; T lymphocyte; tolerance; anergy; memory; gene expression; histone acetylation; DNA methylation

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DNA methylation alterations in systemic lupus erythematosus

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13.1 Introduction

Mammalian immune systems are characterized by the generation of balanced populations of different lymphocyte types that not only ensure the recognition and elimination of potentially harmful molecules but also can remember these antigens and thereby facilitate a rapid response to future exposures. This delicate balance is often disrupted and mammals may lack an efficient response to an external attack or may even produce aberrant responses to their own components. Systemic lupus erythematosus (SLE) is an archetypal example of this second group of diseases, involving hyperactivity of the immune system.

SLE is a chronic, autoimmune, inflammatory and systemic disease characterized by the production of autoantibodies against a variety of nuclear antigens, the deposition of immune complexes and subsequent injury in various tissues including the skin, joints and kidney [1]. One proposed mechanism for the development of antibodies involves the existence of exacerbated apoptosis in circulating lymphocytes. This process leads to a display of nuclear and cytosolic antigens that are normally intracellular and which are associated with disturbance of immune tolerance [2].

The course of SLE is highly variable and characterized by repetitive exacerbations and remissions. The range of symptoms can be classified according to the predominantly affected tissue. Dermatological damage is a typical feature of SLE patients; indeed, this is where the disease's name comes from. People affected by SLE tend to develop a common painless flat red facial rash, also known as a butterfly rash because of its shape, that does not itch but is associated with alopecia. The rash, generally accompanied by ulcers on the

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mucous membrane of the mouth and the nose, is exacerbated by sunlight [3]. SLE patients also suffer musculoskeletal inflammation with consequent arthralgia and myalgia, and associated physical weakness [4]. Multiple haematological cytopenias such as lymphopenia, anaemia, leucopenia or thrombocytopenia, a low level of iron in the blood, functional T cell abnormalities in intracellular signalling and complement system malfunction are common alterations [5]. Cardiovascular and renal systems can also be injured, leading to pericarditis, endocarditis, myocarditis, glomerulonephritis, proteinuria and haematuria, fluid retention and high blood pressure [6]. The circulatory system tends to present inflammation and damage of the blood vessels, increased risk of heart attacks from coronary artery disease and Raynaud's phenomenon [7]: a poor circulation to the fingers and toes with exposure to the cold. Pulmonary manifestations, such as pleuritis and pneumonitis, can also be seen [8]. Finally, neuropsychiatric symptoms, such as depression, psychosis, delirium, meningitis, convulsions, seizures and coma, are serious complications in this autoimmune disease [9].

The prevalence of SLE varies throughout the world between approximately 10 and 50 cases per 100 000 [10]. SLE affects people of all ages and races although there are significant differences between the sexes, ethnic groups and age groups [11]. It is most prevalent in African-American women of fertile age. Furthermore, an impairment of the pathology has been described as being coupled with pregnancy [12]. The highest incidence in women, particularly those of fertile age, has been associated with mechanisms that involve hormones [12] and X-linked genes [13], but not X-inactivation skewing [14]. Additionally, a positive correlation between SLE and Klinefelter's syndrome has been reported [13].

Pathogenesis of SLE

SLE pathogenesis is characterized by exacerbated apoptosis, generation of autoantibodies and aberrant patterns of expression of cytokines in association with loss of immune tolerance and abnormal signal transduction (Figure 13.1) [1]. We currently have no clear understanding of the causes of SLE. This is a matter of considerable debate since efforts are being made to clarify whether some symptoms are in fact a consequence rather than a cause. The loss of immune tolerance to self-components is a major event in the autoimmune process, where dysregulation of gene expression of immune cells and aberrant processing in positive selection are initiating events. As will be discussed below, the inappropriate gene expression may be the result of mutation or altered epigenetic control. Among the genes for which aberrant patterns of gene expression have been reported, remarkable changes are found for cytokine genes (such type I interferon (IFN), which is over-expressed) and signal transduction genes. In relation to cytokines, SLE patients have increased levels of IFN-α in the blood that correlate with disease activity [15]. Regarding cell-signalling pathways, T cells from SLE patients exhibit decreased expression of T cell receptor and interleukin 2 (IL-2), downregulation of protein kinase C-dependent protein phosphorylation and impaired translocation of nuclear factor κB p65 [16].

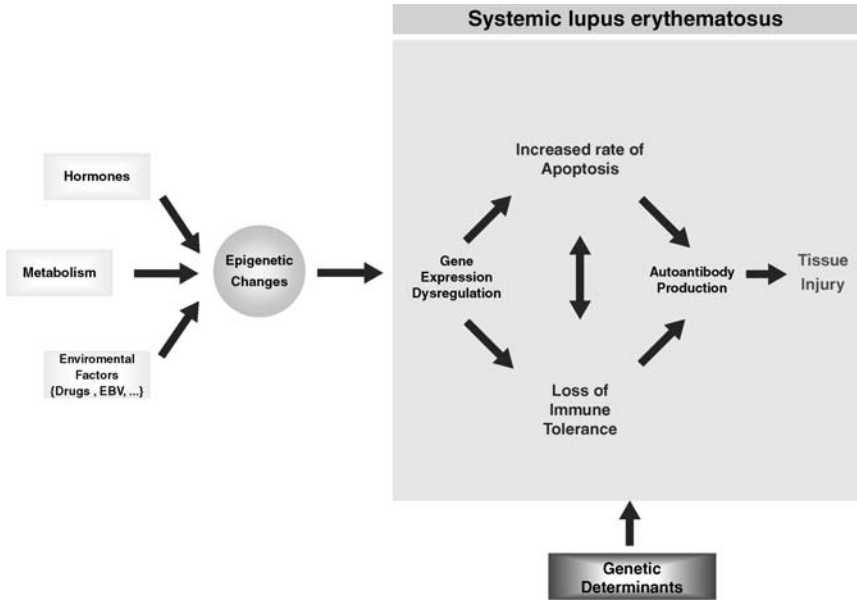

Figure 13.1 A hypothetical diagram summarizing pathogenic mechanisms of SLE and how epigenetic and genetic determinants may be involved in pathogenesis. EBV, Epstein–Barr virus.

In addition to gene expression changes, alterations in cell populations are also found. For instance, many SLE patients exhibit a low proportion of $CD68^+$ macrophages, responsible for the phagocytosis of apoptotic B cells. Interestingly, most of them do not contain apoptotic material. Therefore, dendritic cells of lymph nodes have many autoantigens to present, which leads to the breakdown of immune system tolerance [17].

In SLE, production of antibodies against self-cellular components is a critical feature not only because of its use as a diagnostic tool but also because of it plays and essential role in lupus pathogenesis. In fact, the generation of autoantibodies is responsible for multi-organ damage as a result of inflammatory mechanisms [18]. The accumulation of immune complexes in various tissues is associated with the onset and worsening of the disorder. In SLE patients, autoantibodies of over 50 different specificities have been identified. They can be used for diagnosis purposes, and detection of significant titres of these antibodies can occur years before the onset of the clinical features. For example, anti-La, anti-Ro and anti-phospholipid antibodies typically appear in serum long before any symptoms develop. A peak of anti-Sm and anti-RNP is commonly found just before the illness is manifested. In contrast, anti-nuclear (against histones and nucleosomes), anti-DNA, anti-P, anti-28S rRNA, anti-S10, anti-EBNA-1 and anti-L12 antibodies appear after clinical signs. Anti-P antibodies are the most specific diagnostic marker in SLE because of their absence from any other autoimmune pathologies [18]. However, the presence of anti-nuclear antibodies is the most common marker for diagnosing the disease. In fact, detection

of anti-nuclear antibodies is included in the 11 criteria established by the American College of Rheumatology for SLE diagnosis [19].

Two fundamental events in SLE pathogenesis are both the occurrence of increased rates of apoptosis in monocytes and lymphocytes and the aberrant clearance of dying apoptotic cells [20] (Figure 13.1). Both processes are essential to the release of intracellular autoantigens, which facilitates the immune response. This impaired apoptosis and the loss of immune tolerance contribute to the production of autoantibodies. The main consequences of apoptosis in SLE pathogenesis have been investigated by several experimental approaches. First, injection of apoptotic cells into mice induces autoantibody production. Similarly, increased macrophage apoptosis, induced *in vivo* by the administration of clodronate liposomes, also can exacerbate an autoimmune phenotype [21].

Secondly, apoptotic material activates T and B cells *in vitro* and triggers the release of inflammatory and pro-apoptotic molecules by macrophages. Finally, a relationship between apoptosis rate, increased expression of Fas in B and T cells and symptomatology has been demonstrated. Apoptotic DNA, like bacterial and viral DNA, has a lower 5-methylcytosine (5mC) content than normal or necrotic DNA [22]. Differences in the methylation levels of DNA of apoptotic cells with respect to cells under physiological conditions could also render it more antigenic and exacerbate the immune response [23].

SLE aetiology

The aetiology of SLE is very complex because its development can be induced by multiple factors. Similar to many other autoimmune diseases, SLE demonstrates a complex pattern of inheritance consistent with the involvement of multiple susceptibility genes [24]. During the past few years, multiple candidate genes have been implicated in induction of SLE through association studies, and multiple susceptibility regions have been detected through genome-wide linkage studies. Research based on family studies has reported strong linkage disequilibrium between SLE development and several loci associated with immune response or apoptosis, although there are exceptions. The region 1q23–24, also known as the pentraxin locus, is associated with SLE. The gene that encodes the C-reactive protein (CRP), an important molecule involved in the clearance of apoptotic cell rests, is located in this region [25]. Another remarkable example of gene associated with the disease is the programmed cell death 1 gene (PDCD1). One allele, characterized by a single nucleotide polymorphism that modifies the binding site of a transcription factor, seems to be involved in this pathology [26]. The higher incidence of SLE in women, men with Klinefelter's syndrome and patients with X-linked granulomatosus disease is also of note [14].

In addition to genetic determinants, over 100 substances have been characterized for their drug-induced lupus capacity after months or years of exposure (see below in the section on DNA methylation and drug-induced lupus). This rare adverse immune reaction disappears within weeks of withdrawal of the treatment. Compelling

evidence also demonstrates the causal role of hormones in the development of SLE. As mentioned above, SLE mainly affects women of reproductive age, and the symptoms are exacerbated just before menstruation or by oral contraceptive use [13]. On the other hand, the treatment of SLE mice with male hormones reduces the severity of the disease. Environmental factors, such as microbiological pathogens (e.g. Epstein–Barr virus, EBV) and exposure to chemical elements including mercury, silica and several kinds of pesticides, represent other risk factors for the development of SLE [27]. EBV has been identified as a possible resident virus of SLE B cells, affecting mainly memory cells. Furthermore, patients with active SLE have a higher viral load and stronger virus expression than patients in remission [28]. Also, transgenic introduction of Epstein– Barr nuclear antigen 1 (EBNA-1) into mouse models induces the production of anti-Sm antibodies [29]. The sequence homology between microorganism and self-components can induce crosslinked reactions [30]. Also, LMP2A can subsert signalling pathways [31].

Finally, several lines of evidence indicate the role of environmental triggers in the development and onset of lupus [32]. Environment may induce epigenetic changes, and the studies discussed above suggest that DNA methylation changes are likely to be involved. Much of the current evidence derives from studies of the role of DNA methylation in regulating gene expression in mature T cells. The rest of this chapter discusses the role of epigenetic alterations, with particular emphasis on DNA methylation changes, in the pathoaetiology, diagnosis and treatment of SLE.

13.2 DNA methylation: an epigenetic determinant of lymphocyte function

Cell identity and function result from a combination of genetic determinants and epigenetic regulation. In recent years, epigenetics – the study of stable, reversible and potentially heritable changes in gene expression that do not involve DNA sequence changes [33] – has attracted the attention of many researchers in molecular biology and biomedical research. Epigenetic modifications, cytosine methylation and a variety of post-translational modifications are respectively introduced in DNA and histones (around which DNA is wrapped) to modulate gene levels and to organize nuclear architecture [34,35]. As mentioned above, methylation of DNA in eukaryotes occurs mainly in the 5′ position of cytosines followed by guanines. The introduction of methyl groups in cytosines is catalysed by a group of enzymes named DNA methyltransferases (DNMTs) that use *S*-adenosylmethionine as a donor substrate. CpG dinucleotides are under-represented and, in mammalian genomes, tend to cluster in the promoter of RNA polymerase II-transcribed genes and in repetitive sequences. The CpG-rich regions, also known as CpG islands, tend to be unmethylated and many of them are associated with gene promoters. Relatively few genes are methylated in normal cells, including imprinted genes, genes on the inactive X chromosome and a few tissue-specific genes. Methylation of promoter CpG islands is generally associated with repression of transcription.

Post-translational histone modifications occur in specific sites, generally at the protruding N-termini of histones. Currently, the combination and/or sequential modification of histones are believed to form a 'code' that has specific functional consequences. For instance, lysine hyperacetylation of histones H3 and H4 is associated with an active transcriptional status. Also, trimethylation of Lys-4 of histone H3 is associated with transcriptional activity. However, trimethylation of Lys-9 and -27 of histone H3 is associated with gene repression.

DNA methylation and some histone modifications are mechanistically linked through different nuclear factors, including DNMTs and methyl-CpG-binding domain (MBD) proteins, which not only methylate and recognize methylated DNA, respectively, but also recruit histone-modification enzymes [33].

The increasing interest of biomedical researchers in epigenetic regulation goes hand in hand with the recognition of a number of diseases for which an epigenetic component has been identified. This has been particularly well studied in the context of cancer. However, there are several genetic disorders for which genetic defects in elements of the epigenetic machinery have been identified, such as ICF (immunodeficiency, centromeric instability and facial anomalies) syndrome [36], which features mutations of DNMT3b, and Rett syndrome, for which mutations in the archetypical MBD family member MeCP2 have been described [37,38]. The role of epigenetic regulation is particularly remarkable in the differentiation processes of the haematopoietic system and in association with lymphocyte-activation processes. Here we consider some of these implications and how aberrations in epigenetic regulation are associated with SLE pathogenesis. Furthermore, we raise questions that need to be addressed to unravel the mechanisms underlying these epigenetic alterations.

DNA methylation changes associated with lymphocyte differentiation and activation

The study of epigenetic modifications – DNA methylation and histone modifications – has come to the fore in the field of immune system research. First, as mentioned above, epigenetic regulation has a determinant role in cellular biology as well as in the pathogenesis of immune diseases. Indeed, several key immune processes are likely to be regulated by epigenetic mechanisms. Specifically, sets of genes not required for the function of specific lymphoid cell types may be suppressed by DNA methylation. A clear example is represented by tissue-specific genes. While the extent to which methylation physiologically regulates their expression is unclear, recent examples suggest that it is important for silencing unnecessary genes in specific subsets. On the other hand, active demethylation of genes has been reported for genes that have to be expressed in different lymphocyte subsets. For instance, DNA demethylation of IFN-γ [39] and IL-4 [40] promoters has been associated with the differentiation of naive $CD4^+$ T lymphocytes. Furthermore, active demethylation of the IL-2 promoter occurs in response to activation of these $CD4^+$ T cells. Nevertheless, this interleukin is not expressed in resting naive $CD4^+$ cells due to repressive methylation and the

inactive state of the chromatin [41,42]. In accordance with these findings, cytokines are a key target linking epigenetics and immune development. T cell receptor (TCR) genes expressed in thymocytes are another example. The β locus is methylated when T cells are in the double-negative state, but loses methyl groups as a result of IL-7R signalling. Its demethylation allows DNase activity and subsequent VDJ recombination [43]. CD8α and CD8β are genes that also undergo demethylation, an epigenetic change necessary for the transition of thymocytes from the double-negative to the $CD4^+CD8^+$ state [44]. Antigen presentation and allelic exclusion are other examples. Obviously, lymphocytes are not the only cells that are regulated by epigenetic mechanisms. For example, KIR and Ly49 receptors of natural killer (NK) cells are also associated with this type of control [45].

Although DNMTs are well characterized, the molecular mechanisms causing DNA demethylation are generally poorly understood, with the exception of the passive demethylation caused by DNMT1 inhibition during mitosis. The ability to demethylate DNA actively has been attributed to various factors, including MBD2 [46] and growth arrest and DNA-damage-inducible protein 45a (Gadd45a) [47], a nuclear protein involved in maintaining genomic stability, DNA repair and suppression of cell growth, which may participate in active DNA demethylation. However, unsuccessful attempts by other workers to reproduce the findings of these studies demonstrate that the mechanism of active demethylation is yet to be identified.

13.3 DNA methylation changes in lupus

The importance of epigenetic mechanisms in immune differentiation and function is revealed by immune diseases that are caused at least in part by epigenetic abnormalities. One of the clearest examples is the aforementioned ICF syndrome, which is caused by mutations of the *DNMT3B* gene [36]. This disorder is characterized by B cell immunodeficiency and indicates that genetic abnormalities in the establishment of DNA methylation patterns may contribute to immune disorders. However, epigenetic marks in mature cells must be replicated during mitosis. The maintenance of epigenetic marks on DNA and histones is susceptible to modification by environmental factors, and such changes can lead to aberrant gene expression and abnormal immune function. At present, the role of acquired epigenetic abnormalities in the development of autoimmunity has been studied in human SLE, although other diseases may also have an epigenetic basis. The role of epigenetic alterations, and in particular DNA methylation changes, in the development of SLE is discussed below.

DNA methylation and T cell autoreactivity

Early studies used the irreversible DNMT inhibitor 5-azacytidine (5-azaC) to probe for functional changes caused by DNA methylation inhibition in mature T cells.

One observation was that $CD4^+$ T cells become autoreactive following 5-azaC treatment. $CD4^+$ T cells normally respond to peptides presented in the antigen-binding cleft of self class II major histocompatibility complex (MHC) molecules on antigen-presenting cells. Following 5-azaC treatment, antigen-specific $CD4^+$ T cells lose the requirement for specific antigen and respond solely to antigen-presenting cells. The response is specific for self class II MHC molecules and is reversible, in that antigen responsiveness is slowly recovered once the DNMT inhibitor is removed. The autoreactivity has been demonstrated with cloned and polyclonal human and murine $CD4^+$ T cells [48]. $CD8^+$ T cells do not become autoreactive, although the reason is unknown [49,50]. Mechanistic studies revealed that the autoreactivity is correlated with greater expression of lymphocyte function-associated antigen-1 (LFA-1) (CD11a/CD18), caused by increased levels of CD11a (*ITGAL*) transcripts [51], and LFA-1 over-expression caused by transfection results in identical autoreactivity in human [52] and murine [53] T cells. Bisulphite sequencing revealed demethylation of Alu repeats upstream of the *ITGAL* promoter, and cassette methylation of the region suppressed promoter function in transfection studies [54], implying transcriptional relevance. LFA-1 is an adhesion molecule and surrounds the TCR to form the 'immunological synapse' during activation by antigen-presenting cells, providing both increased stability to the TCR–MHC interaction and co-stimulatory signals [55]. Increased LFA-1 expression may cause a T cell response to MHC molecules that present inappropriate antigens, either by overstabilizing the lower-affinity interaction between the TCR and class II MHC molecules bearing inappropriate peptide fragments, by providing increased co-stimulatory signals or by a combination of the two mechanisms.

DNA demethylation and autoimmunity

The response of demethylated $CD4^+$ cells to self class II MHC molecules demonstrates that normal, antigen-reactive T cells may be modified by exogenous agents to become autoreactive and potentially to contribute to an autoimmune disease. $CD4^+$ T cells similarly responding to host class II MHC molecules cause chronic graft versus host disease (GVHD), with many features of human lupus, including anti-nuclear antibodies and an immune-complex kidney disease [56]. This implies that the 5-azaC-modified cells may cause a disease resembling SLE. Pathogenicity of the autoreactive cells was demonstrated by injecting cloned or polyclonal 5-azaC-treated $CD4^+$ T cells into syngeneic mice. The recipients developed anti-DNA antibodies and an immune-complex glomerulonephritis in addition to other histological features of autoimmunity, depending on the mouse strain [57,58]. LFA-1-transfected $CD4^+$ T cells caused a similar lupus-like disease in the same system, indicating that LFA-1 over-expression contributes to the autoimmunity induced by demethylated T cells [53]. T cell effector functions are also modified by DNA demethylation. Coculture of 5-azaC-treated T cells with autologous B cells results in IgGs hypersecretion [50], due to increased expression of Th1 and Th2 cytokines including IFN-γ, IL-4 and IL-6 [58] and over-expression

of B cell co-stimulatory molecules including CD70 and CD40L [59,60]. IgGs over-stimulation may contribute to the increased autoantibody titres found in mice receiving demethylated T cells. In contrast to the B cell stimulation, coculture of demethylated T cells with autologous monocytes/macrophages (Mø) results in Mø death by apoptosis [52]. Antigen-presenting Mø normally undergo apoptosis after stimulating T cells [61]. However, hypomethylated autoreactive $CD4^+$ T cells respond to all Mø-bearing autologous class II MHC molecules, giving rise to promiscuous Mø death. Increased Mø apoptosis may initiate an anti-DNA response by providing a source of antigenic nucleosomes, since injecting apoptotic cells into normal mice also results in anti-DNA antibodies [62]. Further, transgenic mice lacking one or more of the molecules involved in clearing apoptotic debris develop a lupus-like disease with similar anti-DNA antibodies [63]. The consequence of increased Mø apoptosis has been tested by injecting control and lupus-prone mice with clodronate vesicles. These vesicles are phagocytosed by Mø, causing release of the clodronate and apoptotic death of the phagocyte. Clodronate vesicles give rise to anti-nucleosome antibodies when injected into normal mice and accelerate autoimmunity in lupus-prone mice [64].

DNA methylation and drug-induced lupus

The experiments summarized above imply that inhibiting $CD4^+$ T cell DNA methylation may cause a lupus-like disease. Over 100 drugs have been reported to cause a lupus-like disease with anti-nuclear antibodies, although the generation of autoantibodies is generally confined to a small subset of these patients [65]. In contrast, most patients receiving the anti-arrhythmic agent procainamide or the antihypertensive drug hydralazine eventually develop anti-nuclear antibodies, and a subset of these give rise to an illness resembling lupus [66,67]. These considerations have prompted studies to select procainamide and hydralazine for DNA methylation analysis. Studies *in vitro* showed that both drugs reduced total T cell 5mC content and induced LFA-1 over-expression and autoreactivity in a similar manner to 5-azaC, although the latter was considerably more potent [68]. Procainamide- and hydralazine-treated $CD4^+$ T cells also increased B cell antibody production in a similar manner to 5-azaC-treated T cells [50].

Procainamide was found to be a competitive inhibitor of DNMT enzymic activity and had no effect on intracellular *S*-adenosylmethionine or *S*-adenosylhomocysteine pools [69]. Other studies confirmed that procainamide is a selective DNMT1 inhibitor that reduces the affinity of the enzyme for its substrates, hemimethylated DNA and *S*-adenosylmethionine [70]. In contrast, hydralazine selectively inhibits T cell extracellular-signal-regulated kinase (ERK) pathway signalling, preventing upregulation of DNMT1 and DNMT3a during mitosis, resulting in hypomethylation of the daughter cells [71]. Pathogenicity of decreased ERK pathway signalling was confirmed by treating $CD4^+$ T cells with U0126, a selective MEK inhibitor that reduces ERK pathway signalling, and injecting the cells into syngeneic mice. The T cells over-expressed

LFA-1 and became autoreactive. The mice receiving the treated cells developed anti-DNA antibodies, as observed in procainamide-treated cells [71].

DNA methylation and idiopathic lupus

Similar mechanisms may contribute to idiopathic human lupus. Early studies showed that T cells from patients with active lupus have lower total deoxymethylcytosine content than patients with inactive lupus and normal controls (Figure 13.2). Decreased deoxymethylcytosine content was associated with reduced DNMT enzyme activity [72]. Subsequent studies demonstrated decreased DNMT1 transcripts in lupus T cells [73].

Since lupus T cells have multiple signalling abnormalities [74], and DNMT1 expression is regulated by the ERK and c-Jun N-terminal kinase (JNK) pathways [71], T cell signalling was examined in human lupus. In patients with active lupus (but not those in whom the disease was inactive) the level of ERK phosphorylation dropped in response to stimulation in a manner identical to that seen in hydralazine-treated cells [73], while signalling through the JNK and p38 pathways was unaltered [75]. Other studies revealed functional and epigenetic similarities between lupus and experimentally demethylated T cells. Functional studies showed that lupus T cells overstimulate autologous B cell antibody production, similar to 5-azaC-treated T cells [76]. A subset of lupus T cells also over-expresses LFA-1, and these spontaneously kill autologous Mø in an MHC-restricted, autoreactive fashion, exactly as is observed in experimentally demethylated cells [51]. Further, patients with active lupus have

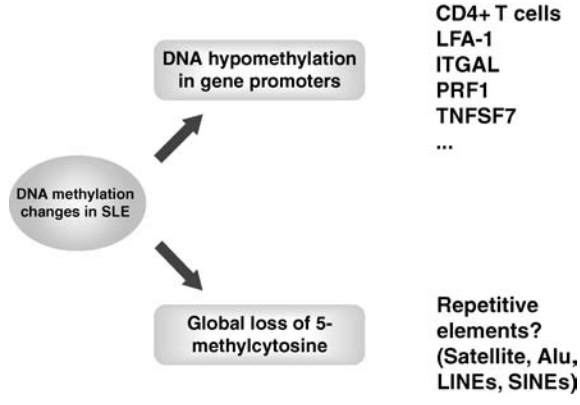

Figure 13.2 DNA methylation changes in SLE $CD4^+$ T cells. Changes described in genes encoding the lymphocyte function-associated antigen-1 (LFA-1), perforin 1 (PRF1) and the tumour necrosis factor (ligand) superfamily, member 7 (TNFSF7, also called CD70) have been associated with transcriptional activation. In addition, changes occur at a global level and show a decrease of the 5-methylcytosine content. Since functional genes represent a small fraction of the genome, it can be speculated that global changes in DNA methylation levels might also occur at repetitive elements of the genome, which are widely represented, including Alu repeats, and short and long interspersed retrotransposable elements (SINEs, LINEs).

circulating apoptotic monocytes in their peripheral blood, suggesting that a similar killing mechanism operates *in vivo* [77]. Epigenetic similarities between 5-azaC-treated and lupus T cells were sought using bisulphite sequencing. As noted above, 5-azaC causes LFA-1 over-expression by demethylating a series of Alu repeats [37] to the *ITGAL* (CD11a) gene (Figure 13.2). Bisulphite sequencing of DNA from $CD4^+$ lupus T cells revealed that the same sequences are demethylated and that this occurs in proportion to the disease activity [70]. The similarities between *ITGAL* demethylation in 5-azaC-treated and lupus T cells raise the possibility that 5-azaC treatment could be used to identify methylation-sensitive T cell genes over-expressed in lupus and to predict which sequences are demethylated. Normal T cells were treated with 5-azaC, affected transcripts were identified using oligonucleotide arrays, and genes relevant to B cell overstimulation and macrophage killing were sought. More than 100 genes were found to be affected, of which perforin (*PRF1*), a cytotoxic molecule normally expressed by NK cells and cytotoxic $CD8^+$ cells but not $CD4^+$ cells, and CD70 (*TNFSF7*), a B cell co-stimulatory molecule, were selected for further study. Studies confirmed that 5-azaC induced perforin mRNA and protein expression in the $CD4^+$ T cell subset, and that expression was due to demethylation of a conserved region located between the *PRF1* promoter and the upstream enhancer [78]. Studies in lupus patients demonstrated identical demethylation of the same sequence and aberrant perforin expression in $CD4^+$ T cells [79]. Concanamycin, a perforin antagonist, prevented the autoreactive Mø killing by lupus T cells, implicating perforin in this phenomenon [64]. Similar studies compared *TNFSF7* methylation, expression and function in 5-azaC-treated and lupus T cells. Bisulphite sequencing revealed that the core *TNFSF7* promoter is normally demethylated in $CD4^+$ T cells and that 5-azaC extends the demethylated region upstream by approximately 300 bp. Cassette methylation confirmed that transcription is suppressed when the region is methylated. Interestingly, other DNA-methylation inhibitors, including procainamide, hydralazine and the mitogen-activated protein kinase/ERK kinase (MEK) inhibitor U0126, all demethylated the same sequence and increased CD70 expression, as occurs with 5-azaC. $CD4^+$ T cells from lupus patients showed CD70 over-expression and demethylation of the same sequence [59,80]. Thus, for at least three genes (*ITGAL*, *TNFSF7* and *PRF1*), identical changes in methylation and expression are found in experimentally demethylated and lupus T cells (Figure 13.2).

Histone modifications in lupus

The role of histone modifications in lupus is less well understood. Treating lupus T cells with histone deacetylase (HDAC) inhibitors including trichostatin A and suberoylanilide hydroxamic acid restores aberrant expression of some genes [81]. For instance, SLE T lymphocytes weakly express IFN-γ and over-express IL-10 and CD40 ligand (CD154) [82]. Although these compounds reverse the histone acetylation status, they also modify acetylation of other proteins, including transcription factors [83]. Confirmatory studies at the chromatin level still need to be performed.

MicroRNAs in lupus

Over the past few years, microRNAs (miRNAs), a new group of gene-expression regulators, has attracted increasing attention. miRNAs are non-coding RNA molecules of around 22 nucleotides in length that regulate the expression of target genes through various post-transcriptional mechanisms [84]. The finding that a set of miRNAs are differentially expressed in SLE patients with respect to normal controls, and the recent description of epigenetic mechanisms, including DNA methylation-dependent regulation of miRNA expression, suggest a role for DNA methylation control of miRNA expression in lupus [85]. miRNAs are therefore potentially involved SLE pathogenesis as well as being possible diagnostic biomarkers [86].

13.4 Epigenetic regulation as a therapeutic target

There is currently no definite cure for SLE. Current therapeutic treatments relieve symptoms and protect organs by decreasing inflammation and/or the level of autoimmune activity in the body. Several molecules have proven successful in SLE treatment, including non-steroidal anti-inflammatory drugs, corticosteroid, hydroxychloroquine, immunosuppressive medications and rituximab. Other strategies, such as plasmapheresis, anti-cytokine therapies, co-stimulation inhibition, B cell anergy or T cell vaccination have also been proposed for relieving clinical symptoms [1,87]. Compelling evidence for the role of epigenetic dysregulation events, which are potentially reversible, in the development of SLE opens up the possibility of using them as targets for the treatment of this disease. In fact, epigenetic drugs are a promising source of therapy for this illness. Many molecules characterized for their ability to modulate the activity of the epigenetic machinery are already being used in preclinical and clinical phases in the treatment of haematological malignancies [88]. Some of them have already received US Food and Drug Administration approval [89]. Before designing a therapeutic approach, it is necessary to have a detailed understanding of the type and extent of epigenetic alterations associated with the disease. Moreover, progress needs to be made to improve and characterize murine models for compound testing. In summary, research effort, adequate funding and a global perspective are fundamental requirements for the successful treatment of SLE [90]. As far as DNA methylation is concerned, it is unlikely that DNMT inhibitors could be used in therapy, since lupus $CD4^+$ T cells are already deficient in 5mC. Rather, identification of the enzymes responsible for active demethylation and of their precise contribution to demethylation processes in T cell activation could contribute to the design of novel compounds of potential use in SLE treatment. On the other hand, drugs targeting the reversal of histone modifications constitute a promising source for SLE therapy. HDAC inhibitors such as trichostatin A or suberoylanilide hydroxamic acids (Vorinostat®) have been shown to reverse the aberrant expression levels of several genes in T cells isolated from MRL-*lpr/lpr* mice. Research into treatment with trichostatin A and suberoylanilide hydroxamic acid shows that these drugs are able to reverse the skewed

gene expression associated with this disorder. Moreover, other HDAC inhibitors, such as the benzamide derivative MS275, have selective anti-inflammatory and specific immune-modulator activity [91,92]. Combination of epigenetic treatment with classical anti-inflammatory therapy could relegate this disorder to the ranks of an asymptomatic condition.

13.5 Future aims for epigenetic research into lupus

As highlighted above, the role of epigenetic dysregulation in SLE pathogenesis has become clear although the extent and number of targets still needs to be accurately described. So far, abnormalities in the DNA methylation patterns in lymphocyte populations are best studied through experimental demethylation of T cells *in vitro*. Identical changes occur at the DNA, mRNA, protein and functional levels for at least three genes that contribute to T cell autoreactivity, B cell overstimulation and macrophage killing (*ITGAL*, *TNFSF7* and *PRF1*), respectively. Experimentally demethylated T cells cause a lupus-like disease *in vivo*. Finally, at least two lupus-inducing drugs are DNA methylation inhibitors with identical effects on T cells to those produced by 5-azaC. In lupus, DNA demethylation appears to be caused by a failure to upregulate DNMT1 during mitosis, due to a defect in ERK pathway signalling. It seems reasonable to propose that defective T cell DNA methylation contributes to the pathogenesis of lupus in genetically predisposed individuals. A more accurate description of the genes that undergo aberrant epigenetic changes, and of the timing and hierarchy of these events, is needed. Establishing the associations between SLE development and epigenetic modifications is another important objective. This knowledge will allow the relevant genes for each step of SLE pathogenesis to be identified. Multifaceted techniques and experimental models are crucial to the achievement of these aims. The bisulphite method coupled with PCR techniques and array platforms is a powerful methodology for DNA methylation analysis. Furthermore, MeDIP, a selective immune-precipitation based on antibodies against methylated cytosines, is a suitable experimental approach for sequence selection [93]. Regarding histone modifications, chromatin immunoprecipitation in conjunction with microarray hybridization (ChIP-on-chip) and ChIP coupled with high-throughput sequencing, using specific antibodies against different histone modifications will be key approaches to the elucidation of sequential changes in the histone-modification profile and the extent of gene alteration [94]. The large amount of data generated from genome-wide experiments will only be useful as long as productive collaborations with bioinformaticians occur. The knowledge that we hope to gain in the near future will help us understand other autoimmune diseases. Finally, development of effective epigenetic therapies is a much longer-term aim since successful treatment can only be based on complete understanding of the disease. All these objectives should be closely coordinated, given that many laboratories will be involved in the work. For this reason, we would expect the International Human Epigenome Project to play a very influential role [95]. We still have a long way to go, but in the near future the epigenetic

determinants of SLE should prove to be of enormous value in the understanding and treatment of the disease.

13.6 Acknowledgements

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References

1. D'Cruz, D.P., Khamashta, M.A. and Hughes, G.R. (2007) Systemic lupus erythematosus. *Lancet* **369**, 587–596.
2. Andrade, F., Casciola-Rosen, L. and Rosen, A. (2000) Apoptosis in systemic lupus erythematosus. Clinical implications. *Rheumatic Disease Clinics of North America* **26**, 215–227.
3. McCauliffe, D.P. and Sontheimer, R.D. (1993) Dermatologic manifestations of rheumatic disorders. *Primary Care* **20**, 925–941.
4. Rothfield, N., Sontheimer, R.D. and Bernstein, M. (2006) Lupus erythematosus: systemic and cutaneous manifestations. *Clinical Dermatology* **24**, 348–362.
5. Keeling, D.M. and Isenberg, D.A. (1993) Haematological manifestations of systemic lupus erythematosus. *Blood Reviews* **7**, 199–207.
6. Smith, D.M., Fortune-Faulkner, E.M. and Spurbeck, B.L. (2000) Lupus nephritis: pathophysiology, diagnosis, and collaborative management. *Nephrology Nursing Journal* **27**, 199–204, 209–211.
7. Yeh, T.-T., Yang, Y.-H., Lin, Y.-T. *et al.* (2007) Cardiopulmonary involvement in pediatric systemic lupus erythematosus: a twenty-year retrospective analysis. *Journal of Microbiology, Immunology and Infection* **40**, 525–531.
8. Kasparian, A., Floros, A., Gialafos, E. *et al.* (2007) Raynaud's phenomenon is correlated with elevated systolic pulmonary arterial pressure in patients with systemic lupus erythematosus. *Lupus* **16**, 505–508.
9. Illei, G.G. (2007) The clinical impact of neuropsychiatric manifestations in early systemic lupus erythematosus. *Nature Clinical Practice Rheumatology* **3**, 428–429.
10. Uramoto, K.M., Michet, Jr, C.J., Thumboo, J. *et al.* (1999) Trends in the incidence and mortality of systemic lupus erythematosus. *Arthritis & Rheumatism* **42**, 46–50.
11. Danchenko, N., Satia, J.A. and Anthony, M.S. (2006) Epidemiology of systemic lupus erythematosus: a comparison of worldwide disease burden. *Lupus* **15**, 308–318.
12. Cooper, G.S., Dooley, M.A., Treadwell, E.L. *et al.* (2002) Hormonal and reproductive risk factors for development of systemic lupus erythematosus: results of a population-based, case-control study. *Arthritis & Rheumatism* **46**, 1830–1839.
13. Chagnon, P., Schneider, R., Hébert, J. *et al.* (2006) Identification and characterization of an Xp22.33;Yp11.2 translocation causing a triplication of several genes of the pseudoautosomal region 1 in an XX male patient with severe systemic lupus erythematosus. *Arthritis & Rheumatism* **54**, 1270–1278.
14. Huang, Q., Parfitt, A., Grennan, D.M. *et al.* (1997) X-chromosome inactivation in monozygotic twins with systemic lupus erythematosus. *Autoimmunity* **26**, 85–93.
15. Hua, J., Kirou, K., Lee, C. *et al.* (2006) Functional assay of type I interferon in systemic lupus erythematosus plasma and association with anti-RNA binding protein autoantibodies. *Arthritis & Rheumatism* **54**, 1906–1916.
16. Fujii, Y., Fujii, K., Tanaka, Y. (2006) Attempt to correct abnormal signal transduction in T lymphocytes from systemic lupus erythematosus patients. *Autoimmunity Reviews* **5**, 143–144.

17. Baumann, I., Kolowos, W., Voll, R.E. *et al.* (2002) Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus. *Arthritis & Rheumatism* **46**, 191–201.
18. Arbuckle, M.R., McClain, M.T., Rubertone, M.V. *et al.* (2003) Development of autoantibodies before the clinical onset of systemic lupus erythematosus. *New England Journal of Medicine* **349**, 1526–1533.
19. American College of Rheumatology (1999) The American College of Rheumatology nomenclature and case definitions for neuropsychiatric lupus syndromes. *Arthritis & Rheumatism* **42**, 599–608.
20. Munoz, L.E., Gaipl, U.S., Franz, S. *et al.* (2005) SLE–a disease of clearance deficiency? *Rheumatology* **44**, 1101–1107.
21. Denny, M.F., Chandaroy, P., Killen, P.D. *et al.* (2006) Accelerated macrophage apoptosis induces autoantibody formation and organ damage in systemic lupus erythematosus. *Journal of Immunology* **176**, 2095–2104.
22. Boix-Chornet, M., Fraga, M.F., Villar-Garea, A. *et al.* (2006) Release of hypoacetylated and trimethylated histone H4 is an epigenetic marker of early apoptosis. *Journal of Biological Chemistry* **281**, 13540–13547.
23. Wen, Z.K., Xu, W., Xu, L. *et al.* (2007) DNA hypomethylation is crucial for apoptotic DNA to induce systemic lupus erythematosus-like autoimmune disease in SLE-non-susceptible mice. *Rheumatology* **46**, 1796–1803.
24. Nath, S.K., Kilpatrick, J., Harley, J.B. (2004) Genetics of human systemic lupus erythematosus: the emerging picture. *Current Opinion in Immunology* **16**, 794–800.
25. Russell, A.I., Cunninghame Graham, D.S., Shepherd, C. *et al.* (2004) Polymorphism at the C-reactive protein locus influences gene expression and predisposes to systemic lupus erythematosus. *Human of Molecular Genetics* **13**, 137–147.
26. Prokunina, L., Castillejo-López, C., Oberg, F. *et al.* (2002) A regulatory polymorphism in PDCD1 is associated with susceptibility to systemic lupus erythematosus in humans. *Nature Genetics* **32**, 666–669.
27. Parks, C.G. and Cooper, G.S. (2006) Occupational exposures and risk of systemic lupus erythematosus: a review of the evidence and exposure assessment methods in population- and clinic-based studies. *Lupus* **15**, 728–736.
28. Moon, U.Y., Park, S.J., Oh, S.T. *et al.* (2004) Patients with systemic lupus erythematosus have abnormally elevated Epstein-Barr virus load in blood. *Arthritis Research & Therapy* **6**, R295–R302.
29. Sundar, K., Jacques, S., Gottlieb, P. *et al.* (2004) Expression of the Epstein-Barr virus nuclear antigen-1 (EBNA-1) in the mouse can elicit the production of anti-dsDNA and anti-Sm antibodies. *Journal of Autoimmunity* **23**, 127–140.
30. Hasler, P. and Zouali, M. (2003) Subversion of B lymphocyte signaling by infectious agents. *Genes and Immunity* **4**, 95–103.
31. Wang, H., Nicholas, M.W., Conway, K.L. *et al.* (2006) EBV latent membrane protein 2A induces autoreactive B cell activation and TLR hypersensitivity. *Journal of Immunology* **177**, 2793–2802.
32. Ballestar, E., Esteller, M. and Richardson, B.C. (2006) The epigenetic face of systemic lupus erythematosus. *Journal of Immunology* **176**, 7143–7147.
33. Wolffe, A.P. and Matzke, M.A. (1999) Epigenetics: regulation through repression. *Science* **286**, 481–486.
34. Fisher, A.G. (2002) Cellular identity and lineage choice. *Nature Reviews Immunology* **2**, 977–982.
35. Espada, J. and Esteller, M. (2007) Epigenetic control of nuclear architecture. *Cellular and Molecular Life Science* **64**, 449–457.
36. Hansen, R.S., Wijmenga, C., Luo, P. *et al.* (1999) The DNMT3B DNA methyltransferase gene is mutated in the ICF immunodeficiency syndrome. *Proceedings of the National Academy of Sciences USA* **96**, 14412–14417.
37. Wilson, A.S., Power, B.E. and Molloy, P.L. (2007) DNA hypomethylation and human diseases. *Biochimica et Biophysica Acta* **1775**, 138–162.
38. Ballestar, E., Ropero, S., Alaminos, M. *et al.* (2005) The impact of MECP2 mutations in the expression patterns of Rett syndrome patients. *Human Genetics* **116**, 91–104.
39. Young, H.A., Ghosh, P., Ye, J. *et al.* (1994) Differentiation of the T helper phenotypes by analysis of the methylation state of the IFN-gamma gene. *Journal of Immunology* **153**, 3603–3610.

40. Lee, D.U., Agarwal, S. and Rao, A. (2002) Th2 lineage commitment and efficient IL-4 production involves extended demethylation of the IL-4 gene. *Immunity* **16**, 649–660.
41. Bruniquel, D. and Schwartz, R.H. (2003) Selective, stable demethylation of the interleukin-2 gene enhances transcription by an active process. *Nature Immunology* **4**, 235–240.
42. Ward, S.B., Hernandez-Hoyos, G., Chen, F. *et al.* (1998) Chromatin remodeling of the interleukin-2 gene: distinct alterations in the proximal versus distal enhancer regions. *Nucleic Acids Research* **26**, 2923–2934.
43. Chattopadhyay, S., Whitehurst, C.E., Schwenk, F. *et al.* (1998) Biochemical and functional analyses of chromatin changes at the TCR-beta gene locus during CD4-CD8- to CD4 + CD8 + thymocyte differentiation. *Journal of Immunology* **160**, 1256–1267.
44. Carbone, A.M., Marrack, P. and Kappler, J.W. (1988) Demethylated CD8 gene in CD4 + T cells suggests that CD4 + cells develop from CD8 + precursors. *Science* **242**, 1174–1176.
45. Chan, H.W., Kurago, Z.B. and Stewart, C.A. *et al.* (2003) DNA methylation maintains allele-specific KIR gene expression in human natural killer cells. *Journal of Experimental Medicine* **197**, 245–255.
46. Bhattacharya, S.K., Ramchandani, S., Cervoni, N. *et al.* (1999) A mammalian protein with specific demethylase activity for mCpG DNA. *Nature* **397**, 579–583.
47. Barreto, G., Schäfer, A., Marhold, J. *et al.* (2007) Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation. *Nature* **445**, 671–675.
48. Richardson, B. (2003) DNA methylation and autoimmune disease. *Clinical Immunology* **109**, 72–79.
49. Richardson, B. (1986) Effect of an inhibitor of DNA methylation on T cells. II. 5-Azacytidineinduces self-reactivity in antigen-specific T4 + cells. *Human Immunology* **17**, 456–470.
50. Richardson, B.C., Liebling, M.R. and Hudson, J.L. (1990) CD4 + cells treated with DNA methylation inhibitors induce autologous B cell differentiation. *Clinical Immunology and Immunopathology* **55**, 368–381.
51. Richardson, B.C., Strahler, J.R., Pivirotto, T.S. *et al.* (1992) Phenotypic and functional similarities between 5-azacytidine treated T cells and a T cell subset in patients with active systemic lupus erythematosus. *Arthritis & Rheumatism* **35**, 647–662.
52. Richardson, B., Powers, D., Hooper, F. *et al.* (1994) Lymphocyte function-associated antigen 1 overexpression and T cell autoreactivity. *Arthritis & Rheumatism* **37**, 1363–1372.
53. Yung, R., Powers, D., Johnson, K. *et al.* (1996) Mechanisms of drug-induced lupus. II. T cells overexpressing lymphocyte function-associated antigen 1 become autoreactive and cause a lupus-like disease in syngeneic mice. *Journal of Clinical Investigation* **97**, 2866–2871.
54. Lu, Q., Kaplan, M., Ray, D. *et al.* (2002) Demethylation of ITGAL (CD11a) regulatory sequences in systemic lupus erythematosus. *Arthritis & Rheumatism* **46**, 1282–1291.
55. Wülfing, C., Sumen, C., Sjaastad, M.D. *et al.* (2002) Costimulation and endogenous MHC ligands contribute to T cell recognition. *Nature Immunology* **3**, 42–47.
56. Rolink, A.G., Pals, S.T. and Gleichmann, E. (1983) Allosuppressor and allohelper T cells in acute and chronic graft-vs.-host disease. II. F1 recipients carrying mutations at H-2Kand/or I-A. *Journal of Experimental Medicine* **157**, 755–771.
57. Yung, R.L., Quddus, J., Chrisp, C.E. *et al.* (1995) Mechanism of drug-induced lupus. I. Cloned Th2 cells modified with DNA methylation inhibitors in vitro cause autoimmunity in vivo. *Journal of Immunology* **154**, 3025–3035.
58. Quddus, J., Johnson, K.J. Gavalchin, J. *et al.* (1993) Treating activated CD4 + T cells with either of two distinct DNA methyltransferase inhibitors 5-azacytidine or procainamide, is sufficient to cause a lupus-like disease in syngeneic mice. *Journal of Clinical Investigation* **92**, 38–53.
59. Oelke, K., Lu, Q., Richardson, D. *et al.* (2004) Overexpression of CD70 and overstimulation of IgGs synthesis by lupus T cells and T cells treated with DNA methylation inhibitors. *Arthritis & Rheumatism* **50**, 1850–1860.
60. Lu, Q. (2006) Women and lupus: the inactive X awakens. *Arthritis & Rheumatism* **54**, S775.
61. Richardson, B.C., Buckmaster, T., Keren, D.F. *et al.* (1993) Evidence that macrophages are programmed to die after activating autologous, cloned, antigen-specific, CD4 + T cells. *European Journal of Immunology* **23**, 1450–1455.

62. Mevorach, D., Zhou, J.L., Song, X. *et al.* (1998) Systemic exposure to irradiated apoptotic cells induces autoantibody production. *Journal of Experimental Medicine* **188**, 387–392.
63. Walport, M.J. (2000) Lupus, DNase and defective disposal of cellular debris. *Nature Genetics* **25**, 135–136.
64. Denny, M.F., Chandaroy, P., Killen, P.D. *et al.* (2006) Accelerated macrophage apoptosis induces autoantibody formation and organ damage in systemic lupus erythematosus. *Journal of Immunology* **176**, 2095–2104.
65. Yung, R. and Richardson, B. (2003) Drug-induced lupus. In: Hochberg M., Silman A., Smolen J. *et al.* (eds), Rheumatology, p. 1385 Mosby, New York.
66. Henningsen, N.C., Cederberg, A., Hanson, A. *et al.* (1975) Effects of long-term treatment with procaine amide. A prospective study with special regard to ANF and SLE in fast and slow acetylators. *Acta Medica Scandinavica* **198**, 475–482.
67. Blomgren, S.E., Condemi, J.J. and Vaughan, J.H. (1972) Procainamide-induced lupus erythematosus. Clinical and laboratory observations. *American Journal of Medicine* **5**, 338–348.
68. Cornacchia, E., Golbus, J., Maybaum, J. *et al.* (1988) Hydralazine and procainamide inhibit T cell DNA methylation and induce autoreactivity. *Journal of Immunology* **140**, 2197–2200.
69. Scheinbart, L.S., Johnson, M.A., Gross, L.A. *et al.* (1991) Procainamide inhibits DNA methyltransferase in a human T cell line. *Journal of Rheumatology* **18**, 530–534.
70. Lee, B.H., Yegnasubramanian, S., Lin, X. *et al.* (2005) Procainamide is a specific inhibitor of DNA methyltransferase 1. *Journal of Biological Chemistry* **280**, 40749–40756.
71. Deng, C., Lu, Q., Zhang, Z. *et al.* (2003) Hydralazine may induce autoimmunity by inhibiting extracellular signal- regulated kinase pathway signaling. *Arthritis & Rheumatism* **48**, 746–756.
72. Richardson, B., Scheinbart, L., Strahler, J. *et al.* (1990) Evidence for impaired T cell DNA methylation in systemic lupus erythematosus and rheumatoid arthritis. *Arthritis & Rheumatism* **33**, 1665–1673.
73. Deng, C., Kaplan, M.J., Yang, J. *et al.* (2001) Decreased Ras-mitogen-activated protein kinase signaling may cause DNA hypomethylation in T lymphocytes from lupus patients. *Arthritis & Rheumatism* **44**, 397–407.
74. Kammer, G.M., Perl, A., Richardson, B.C. *et al.* (2002) Abnormal T cell signal transduction in systemic lupus erythematosus. *Arthritis & Rheumatism* **46**, 1139–1154.
75. Oelke, K. and Richardson, B. (2004) Decreased T cell ERK pathway signaling may contribute to the development of lupus through effects on DNA methylation and gene expression. *International Reviews of Immunology* **23**, 315–331.
76. Crow, M. (1999) Mechanisms of T-helper cell activation and function in systemic lupus erythematosus, In: Kammer G.M. and Tsokos G. (eds), *Lupus: Molecular and Cellular Pathogenesis*, p. 231 Humana Press, Totowa, NJ.
77. Richardson, B.C., Yung, R.L., Johnson, K.J. *et al.* (1996) Monocyte apoptosis in patients with active lupus. *Arthritis & Rheumatism* **39**, 1432–1434.
78. Lu, Q., Wu, A., Ray, D. *et al.* (2003) DNA methylation and chromatin structure regulate T cell perforin gene expression. *Journal of Immunology* **170**, 5124–5132.
79. Kaplan, M.J., Lu, Q., Wu, A. *et al.* (2004) Demethylation of promoter regulatory elements contributes to perforin overexpression in CD4+ lupus T cells. *Journal of Immunology* **172**, 3652–3661.
80. Lu, Q., Wu, A. and Richardson, B.C. (2005) Demethylation of the same promoter sequence increases CD70 expression in lupus T cells and T cells treated with lupus-inducing drugs. *Journal of Immunology* **174**, 6212–6219.
81. Mishra, N., Brown, D.R., Olorenshaw, I.M. *et al.* (2001) Trichostatin A reverses skewed expression of CD154, interleukin-10, and interferon-gamma gene and protein expression in lupus T cells. *Proceedings of the National Academy of Sciences USA* **98**, 2628–2633.
82. Mishra, N., Reilly, C.M., Brown, D.R. *et al.* (2003) Histone deacetylase inhibitors modulate renal disease in the MRL-lpr/lpr mouse. *Journal of Clinical Investigation* **111**, 539–552.
83. Imhof, A., Yang, X.J., Ogryzko, V.V. *et al.* (1997) Acetylation of general transcription factors by histone acetyltransferases. *Current Biology* **7**, 689–692.
84. Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297.

85. Lujambio, A. and Esteller, M. (2007) CpG island hypermethylation of tumor suppressor microRNAs in human cancer. *Cell Cycle* **6**, 1455–1459.
86. Dai, Y., Huang, Y.S., Tang, M. *et al.* (2007) Microarray analysis of microRNA expression in peripheral blood cells of systemic lupus erythematosus patients. *Lupus* **16**, 939–946.
87. Mount, G.R. and Gilliland, W.R. (2008) Emerging biological therapies in systemic lupus erythematosus. *Clinical Pharmacology & Therapeutics* **83**, 167–171.
88. Villar-Garea, A. and Esteller, M. (2004) Histone deacetylase inhibitors: understanding a new wave of anticancer agents. *International Journal of Cancer* **112**, 171–178.
89. Yoo, C.B. and Jones, P.A. (2006) Epigenetic therapy of cancer: past, present and future. *Nature Reviews Drug Discovery* **5**, 37–50.
90. Fuks, F., Hurd, P.J., Wolf, D. *et al.* (2003) The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *Journal of Biological Chemistry* **278**, 4035–4040.
91. Adcock, I.M. (2007) HDAC inhibitors as anti-inflammatory agents. *British Journal of Pharmacology* **150**, 829–831.
92. Brogdon, J.L., Xu, Y., Szabo, S.J. *et al.* (2007) Histone deacetylase activities are required for innate immune cell control of Th1 but not Th2 effector cell function. *Blood* **109**, 1123–1130.
93. Weber, M., Davies, J.J., Wittig, D. *et al.* (2005) Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nature Genetics* **37**, 853–862.
94. Weinmann, A.S., Yan, P.S., Oberley, M.J. *et al.* (2002) Isolating human transcription factor targets by coupling chromatin immunoprecipitation and CpG island microarray analysis. *Genes & Development* **16**, 235–244.
95. Esteller, M. (2006) The necessity of a human epigenome project. *Carcinogenesis* **27**, 1121–1125.

Abstract

Systemic lupus erythematosus (SLE) is one of the best-studied autoimmune diseases from the point of view of understanding how the acquisition of epigenetic abnormalities associates with the development of autoimmunity. The disease, which primarily affects women, is characterized by the formation of autoantibodies to nuclear antigens and immune-complex deposition in tissues such as the skin and kidney. It has been proposed that SLE arises in genetically predisposed individuals when exposed to one or more environmental triggers. Specifically, environmentally induced epigenetic changes, and in particular altered DNA methylation changes, have been described as being associated with lupus. In this article, we discuss the implications of changes in DNA methylation and other epigenetic alterations with respect to SLE, and how our knowledge of the epigenetic targets in SLE can contribute not only to a better understanding disease pathogenesis, but also to the design of novel and more specific pharmaceutical compounds.

Key words: systemic lupus erythematosus; DNA methylation; epigenetics

14

Long-range histone acetylation patterns in the development of autoimmunity

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14.1 Introduction

In response to antigenic challenge, naïve T cells differentiate in the periphery into effector cells capable of transcribing key cytokine genes that orchestrate the adaptive immune response. One such cytokine gene is the *Ifng* gene, whose expression is essential for cell-mediated immunity against intracellular pathogens. Complex long-range epigenetic modifications, including histone acetylation and deacetylation and histone methylation and demethylation, play essential roles in this differentiation process allowing effector T cells to efficiently transcribe *Ifng*. The purpose of this review is to illustrate the dynamic nature of these epigenetic modifications during the differentiation process and show how defects in their establishment result in ectopic expression of *Ifng* that may contribute to autoimmunity.

14.2 The histone code hypothesis

Chromatin structure comprises a repeating unit of histones and about 200 bp of DNA. The N-terminal 'tails' of core histones, H2A, H2B, H3 and H4, are subjected to a variety of post-translational modifications including methylation, acetylation, phosphorylation, ubiquitination and ADP-ribosylation [1]. Specific enzymes catalyse

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these modifications [2]. The recognition that deletion or inhibition of these enzymes changes activation and silencing of specific genes has given rise to the histone code hypothesis, which proposes that the epigenetic modification of histones at specific gene loci is a key element in the acquisition and maintenance of developmental and cell-type-specific expression and silencing of genes [3]. The general view is that these marks are passed on to daughter cells after division and thus represent a heritable change to the cell, hence the term epigenetic modification [4–6].

Enzymes that catalyse these histone modifications do not directly bind to DNA [3,7,8]. Rather, these proteins have binding sites for a whole variety of different transcription factors and other DNA-binding proteins and are typically recruited to DNA via their ability to bind these transcription factors rather than their ability to bind directly to DNA. In some instances these large complexes are composed of additional associated proteins required for enzymic activity. Specific enzymes also catalyse deacetylation (histone deacetylases, HDACs) and demethylation (histone demethylases) of specific histones. Thus the level of a histone acetylation at a specific site in the genome reflects the balance between the level of histone acetyltransferases (HATs) and HDAC recruited to a given genomic site. Similarly, the level of histone methylation reflects the balance between histone lysine methyltransferase activity and histone lysine demethylase activity at a genomic site. The discovery of histone lysine demethylases has markedly altered the landscape of the histone code [9,10]. Previously, the general view was that histone methylation marks (see next paragraph) are stable marks but the identification of histone lysine demethylases indicates that these marks may be highly dynamic.

Evidence also supports the idea that multiple histone marks may impart additional information to the cell. For example, plasticity of embryonic stem cells may be established via the histone code. Specific developmentally regulated genes are marked by large domains of repressive methylation marks superimposed upon smaller domains of activating methylation marks [11]. By this mechanism, removal of repressive marks in cells where the gene needs to be expressed may facilitate transcription and removal of activating marks would facilitate repression of transcription in cells that are not supposed to express a given gene. An additional layer of complexity is produced by the possibility of acquiring mono-, di-, or trimethyl marks at the different lysine residues in the H3 and H4 histones [12].

A surprising fact that emerged from a variety of studies performed over recent years is the recognition that very large domains of histone marks are established during developmental activation or silencing of genes that extend many kilobases beyond the coding regions of genes [13–21]. A likely explanation is that these domains are centred on genomic sequences exhibiting strong evolutionary conservation (conserved sequences, CNS) [22,23]. It has been argued that this sequence conservation implies a functional role and this is borne out by experience. In fact, CNS very distant from a given gene play active roles in transcriptional activation or silencing. Spreading of histone marks beyond the CNS also occurs. This is probably achieved because the histone marks represent high-affinity binding sites for histone-modifying enzymes and this permits progressive spread of these marks across adjacent nucleosomes [17]. Extended domains of histone marks at developmentally regulated genes and gene loci

are observed at all stages of development from embryonic stem cells, as mentioned above, to programming of thymocytes as they progress through their developmental stages, to differentiation of mature T cells as they endure further developmental programmes in the periphery to become effector T helper 1 (Th1) or Th2 cells, which selectively express either the *Ifng* gene or the *Il4* gene, respectively. These extended patterns of histone marks are not only observed in closely linked gene families that exhibit coordinate regulation of expression, such as the globin gene locus, the growth hormone gene locus and the IL-4 gene locus but also in single genes not known to be members of extended gene families, such as the gene that encodes interferon (IFN)-γ. Genes that exhibit these extended domains of histone acetylation generally exhibit both high levels of expression and tissue specific expression patterns. The *Ifng* gene serves as an example. This gene consists of five exons and four introns spanning about 5 kb of genomic DNA and is highly conserved in mammalian species. *Ifng* is expressed predominantly by a small subset of cells of the haematopoietic lineage that include T lymphocytes, natural killer (NK) cells and NKT cells. In these cells, long-range histone H4 acetylation marks span a genomic region that is at least 75 kb upstream and downstream of the *Ifng* gene.

14.3 Epigenetic defects as a mechanism of disease

Epigenetics is defined as information that is heritable but does not arise from the DNA sequence [24]. Two major classes of epigenetic information are represented by DNA methylation and histone modifications as outlined above. Given the critical role epigenetic marks play in normal development, it is worth exploring what is known about epigenetic defects in disease processes. Potentially, epigenetic defects can produce developmental alterations that may result from either inheritance or from environmental cues that affect translation of the epigenetic code [25]. Defects in the epigenetic code could be locus-specific and produced by polymorphisms/mutations in the underlying genetic code. Alternatively, defects in any of the array of proteins involved in establishing the epigenetic code could produce developmental defects leading to human disease. Examples include specific loci where genetic lesions lead to improper gene regulation due to failure to establish the epigenetic code. In Beckwith–Wiederman syndrome, for example, patients have sequence variations and deletions in a region that is normally differentially methylated on different alleles [26]. A second example includes defects in the epigenetic machinery that produce developmental disorders generally representing single-gene defects and relatively rarely encountered in the human population. An example is immunodeficiency, centromeric instability and facial anomalies (ICF) syndrome [27], a condition that affects the immune system and is produced by mutations in the DNA methyltransferase gene, *DNMT3B*, responsible for DNA methylation during development. The syndrome probably arises from failure to form heterochromatin during development. In general, these types of disorders result from mutations in regulators of epigenesis during development, thus producing phenotypic changes in many organ systems.

More common diseases, such as autoimmune diseases, are generally viewed as multigenic diseases that also have an environmental component. Systemic autoimmune diseases may also have epigenetic components. For example, T lymphocytes from patients with systemic lupus erythematosus (SLE) exhibit hypo-DNA methylation of certain genes that play critical functions in the immune system and treatment of mice with drugs, such as 5-azacytidine or procainamide, which inhibit DNA methylation, induces syndromes in mice similar to human SLE [28]. Histone acetylation also seems to play a role in this disease. In mice, deletion of one of the major HATs in B lymphocytes, p300, produces a disorder very similar to human SLE [29]. Selective pharmacological inhibition of HDACs has therapeutic activity in a mouse model of SLE and these same agents correct expression levels of key genes and proteins that exhibit aberrant expression patterns in human SLE [30,31]. Further, overall total levels of specific histone marks are different in inbred strains of mice that develop lupus-like diseases compared to control mice [32]. Specifically, histones from splenocytes (~70% B lymphocytes) from mice that develop lupus-like disease exhibit increases in the level of histones that are hypermethylated and decreases in the level of histones that are hyperacetylated. Taken together, these data suggest that lupus-like diseases may be associated with defects in the enzyme complexes that establish the histone code rather than specific genetic defects that alter the way the histone code is established at a given genomic locus. These individual subjects are dealt with in other chapters in this book in greater detail. Thus in lupus, as well as other autoimmune diseases, imbalance in the epigenetic code may contribute to disease onset or pathogenesis and it may be possible to affect the course of disease by altering the epigenetic code through increasing levels of activating histone acetylation marks or decreasing levels of inhibitory histone methylation marks, either generally or at specific genomic loci.

14.4 Analysis of the histone code

Introduction of the chromatin immunoprecipitation (ChIP) assay has had a great impact on the study of epigenetic mechanisms in development and differentiation [33]. Briefly, cells or tissues of interest are crosslinked with paraformaldehyde and chromatin is isolated. Chromatin is sheared to produce small fragments of approximately 500 bp of genomic DNA. Sheared chromatin is immunoprecipitated with the antibody of choice, for example anti-acetylated H4, or control antibody. DNA is purified from the immunoprecipitate. DNA is PCR-amplified with specific primers to quantify the amount of DNA immunoprecipitated with the specific antibody relative to an irrelevant antibody using either semi-quantitative methods (agarose gels) or quantitative methods (real-time PCR) and results are standardized using a variety of methods. With the advent of specific antibodies that recognize modified histone tails, the ChIP assay has produced much of the information demonstrating where histone marks are localized in the genome. Applications range from examination of histone tail modifications at promoters and transcribed regions of genes to large-scale examination

of the localization of specific histone tail modifications across an entire genome in an individual cell type.

14.5 Long-range histone acetylation patterns in Th cell differentiation

$CD4^+$ T cells play a critical role in protection against foreign pathogens through their ability to orchestrate the activities and functions of both the innate and adaptive arms of the immune system. A major mechanism by which this is achieved is through their ability to differentiate in the periphery into effector cells that produce specific cytokines [34,35]. Three broad classes of effector T cells have been described: Th1 cells whose signature cytokine is IFN-γ, Th2 cells whose signature cytokine is IL-4 and Th17 cells whose signature cytokine is IL-17. A general model proposes that naïve T cells have the potential to differentiate into each of these three distinct cell lineages. Early during the differentiation programme, *Il4* and *Ifng* genes are transcribed at similar rates in developing Th1 and Th2 cells [36]. Soon thereafter, transcriptional rates demarcate dramatically and the Th1 differentiation programme leads to marked increases in the transcriptional activity of the *Ifng* gene and silencing of the *Il4*, *Il13* and *Il5* genes. The converse is true for the Th2 differentiation programme, which leads to increased transcriptional activity of *Il4*, *Il5* and *Il13* and silencing of *Ifng*. Key stimulators of the Th1 differentiation programme include the IL-12/signal transducer and activator of transcription 4 (STAT4) and the IFN-γ/STAT1/T-bet signalling pathways and stimulators of the Th2 differentiation programme include IL-4/STAT6 and GATA-3 signalling pathways. Thus, the differentiation of naïve T lymphocytes along either Th1 or Th2 differentiation pathways represents an attractive model with which to examine epigenetic mechanisms leading to activation or silencing of the *Il4*, *Il13* and *Ifng* genes [37–40].

A key element of both Th1 and Th2 differentiation programmes is the acquisition of broad non-uniform histone-acetylation marks across the *Ifng* and *Il4*/*Il13*/*Il5* loci, respectively [15,16,41,42]. In Th1 cells, establishing these histone acetylation marks across the *Ifng* locus is dependent upon both STAT4 and T-bet. Absence of STAT4 largely prevents acquisition of these long-range histone acetylation marks while absence of T-bet prevents acquisition of histone acetylation marks 3′ of the transcribed region of *Ifng*. In addition, mature NK cells, which also transcribe *Ifng* after appropriate stimulation, possess a similar distribution of histone acetylation marks across the entire *Ifng* locus. This broad histone acetylation pattern is established across a linear region of genomic DNA that spans more than 100 kb and each peak of histone acetylation coincides with a CNS. Often, searches for evolutionary conservation is one method to identify promoters of genes. Searches for evolutionary conservation across the *Ifng* gene locus reveal that multiple sequences are conserved that are very distant from the *Ifng* gene and these individual CNS exhibit unique functions. First, rather than having a single binding site at the promoter for STAT4 and T-bet transcription factors that drive

the Th1 differentiation programme, multiple STAT4- and T-bet-binding sites exist across the more than 100 kb *Ifng* locus. Second, many distal elements exhibit strong ability to stimulate transcription in various assays. Third, looping mechanisms are in place to bring these distal elements into close proximity to the gene that should permit physical contact between key transcription factors, the basal transcriptional machinery and components of the epigenetic machinery necessary to install the histone code and achieve high-level transcription [43]. Thus an emerging picture is that genes exhibiting high-level and cell-specific transcription are regulated via a three-dimensional chromatin structure that brings distal activating and repressive elements that bind key transcription factors with the epigenetic machinery to the gene to achieve precise transcriptional control.

Proliferating T cells that are not driven to differentiate along the Th1 pathway do not establish histone acetylation marks across the *Ifng* locus and do not transcribe the *Ifng* gene at high rates [15,44]. Lack of histone acetylation marks across the *Ifng* locus could be due to a lack of recruitment of HATs to the *Ifng* locus or the presence of HDACs across the *Ifng* locus or both. One simple approach to this question is to use a selective HDAC inhibitor, such as trichostatin A, and determine whether T cell activation alone, in the presence of trichostatin A, is sufficient to establish histone acetylation marks across the *Ifng* locus and stimulate *Ifng* transcription in proliferating T cells in the absence of any differentiating stimulus (IL-12) or in the presence of a Th2 differentiation stimulus (IL-4). These analyses demonstrate that inhibition of HDAC activity is sufficient to establish histone acetylation marks across the *Ifng* locus and to stimulate *Ifng* transcription in proliferating T cells, thus arguing that the level of histone acetylation at the *Ifng* locus reflects the balance between recruitment of HATs and HDACs to the locus. This is not the case in Th2 cells, where dominant repressive H3K27-methylation marks are established across the locus, presumably blocking recruitment of HATs to the locus: so simply inhibiting HDACs is insufficient to induce histone acetylation across the locus and stimulate *Ifng* transcription [44]. STAT6 and GATA-3, two transcription factors required for Th2 lineage commitment, are necessary and sufficient to establish repressive H3K27 methylation marks across the *Ifng* locus in effector Th2 cells. A unifying model would be that the balance of HATs and HDACs recruited to the *Ifng* locus in proliferating T cells and Th1 cells determines whether the locus is histone-acetylated or not, and undergoing high or low levels of transcription, respectively; but dominant repressive marks are established at the locus in effector Th2 cells to actively prevent transcription.

A prediction of the above model is that an HDAC complex should be recruited to the *Ifng* locus in proliferating T cells but not in proliferating effector Th1 cells. Histone-deacetylating activity at a genomic locus involves recruitment of a multi-protein complex including an HDAC, the evolutionarily conserved corepressor Sin3A, and Sin3A-associated proteins, such as SAP30 and SAP18 [45,46]. Typically, this complex does not bind directly to DNA but requires a transcription factor to recruit the HDAC complex to DNA. Therefore, localization of Sin3A to the *Ifng* locus can be used as a surrogate for HDAC activity at the locus. The major site of Sin3A occupancy in proliferating T cells is at the −22 kb CNS site upstream of the the *Ifng* promoter

(S. Chang and T.M. Aune, unpublished results). Minor sites are at the promoter and −55 kb and −57 kb from the promoter. Occupancy of the −22 kb CNS site by Sin3A is reversed in effector Th1 cells. Thus, an HDAC complex is recruited to the *Ifng* locus in proliferating T cells at both major and minor sites. Available data suggest that in response to Th1 differentiation conditions the HDAC complex is forced off of the −22 kb site by recruitment of a T-bet–HAT complex leading to dominant formation of long-range histone acetylation marks across the *Ifng* locus. Thus, the histone code is involved extensively in the positive and negative regulation of *Ifng* transcription in T cells.

14.6 Long-range histone acetylation and autoimmunity

The non-obese diabetic (NOD) mouse strain spontaneously develops autoimmune diabetes [47,48]. The disease is characterized by insulitis in the pancreas followed by selective destruction of β cells in pancreatic islets. Evidence suggests that β cell destruction is mediated, at least in part, by effector $CD4^+$ T cells that preferentially secrete IFN-γ and tumour necrosis factor α. NOD $CD4^+$ T cells have been shown to have an increased propensity to become effector Th1 cells in tissue culture assays. Studies in tissue-culture models demonstrate that, in contrast to other murine strains, $CD4^+$ T cells from the autoimmune-prone NOD strain are genetically programmed to utilize IL-2 as a driving cytokine to differentiate into IFN-γ-producing effector T cells [49]. Thus, an antigen stimulus is sufficient to drive both T cell proliferation and Th1 differentiation. This property may represent a liability that contributes to initial tissue inflammatory responses and development of autoimmune diabetes in NOD mice.

The pattern of long-range histone acetylation marks across the *Ifng* locus is relatively similar in effector Th1 cells from C57BL/6, BALB/c and NOD strains [15]. The *Ifng* locus in resting naïve T cells from each of these strains does not harbour detectable long-range histone acetylation marks. In sharp contrast, long-range histone acetylation marks across the entire *Ifng* locus, which are absent in proliferating $CD4^+$ and $CD8^+$ T cells from C57BL/6 and BALB/c strains, are fully established in proliferating $CD4^+$ and $CD8^+$ T cells from the NOD strain coinciding with their ability to transcribe the *Ifng* gene. As in the C57BL/6 strain, acquisition of long-range histone acetylation marks across the *Ifng* locus is blocked in effector Th2 cells from the NOD strain, as is *Ifng* transcription.

These properties of NOD T cells may be consistent with alterations in the balance of HAT or HDAC recruitment to the *Ifng* locus. Either recruitment of increased HAT activity or decreased HDAC activity to the *Ifng* locus could account for the observed differences in the pattern of long-range histone acetylation marks across the locus. However, since long-range histone acetylation across the *Ifng* locus in effector Th1 cells requires the transcription factors T-bet and STAT4 and since differences in expression levels of these transcription factors in NOD T cells have not been observed, it seems unlikely that differences in HAT recruitment to the *Ifng* locus account for the

differences in histone acetylation observed in NOD T cells compared to T cells of other strains [15]. Thus, failure to recruit the HDAC complex to the *Ifng* locus in proliferating NOD T cells may account for the observed increases in long-range histone acetylation at the *Ifng* locus and increased transcription of the *Ifng* gene. In fact, this is what is found. The *Ifng* locus is completely devoid of detectable levels of the HDAC complex in proliferating NOD T cells (S. Chang and T.M. Aune, unpublished results). This is most striking at the −22 kb CNS site where HDAC complexes are heavily recruited in proliferating C57BL/6 T cells but completely absent in proliferating NOD T cells. Thus, increased long-range histone acetylation at the *Ifng* locus in proliferating NOD T cells is most likely due to lack of recruitment of HDAC complexes rather than increased recruitment of HAT complexes. Failure of proliferating NOD T cells to recruit the HDAC complex to the *Ifng* locus may be due to genetic polymorphisms at the −22 kb site between NOD T cells and other strains. However, this seems unlikely since the HDAC–Sin3 complex is recruited to multiple sites across the *Ifng* locus in proliferating C57BL/6 T cells, but there is virtual absence of the HDAC–Sin3 complex in proliferating NOD T cells. Since transcription factors or other DNA-binding proteins are required to tether the HDAC complex to DNA, defects in levels or function of the necessary DNA-binding proteins in NOD T cells may also explain failure of HDAC complexes to be recruited to the *Ifng* locus. A third possibility is that the HDAC complex is defective in NOD T cells. The HDAC complex is a multi-protein complex so defects in any of these activities in NOD T cells may contribute to the observed loss of recruitment of HDAC–Sin3 complexes to the *Ifng* locus in NOD T cells.

Any of the above scenarios could contribute to development of autoimmunity in NOD mice. First, production of IFN-γ by $CD4^+$ and $CD8^+$ T cells is thought to contribute to early phases of β-cell destruction in NOD mice. Under normal conditions, T cells emerge from developmental programmes in the thymus and migrate to peripheral lymphoid organs. Once in the periphery, they must endure further differentiation programmes before they are competent to transcribe key effector cytokine genes, such as the *Ifng* gene, necessary for their function in the immune system. Developmental programmes required to make a T cell competent to transcribe the *Ifng* gene require both antigenic stimulation to drive cell division and clonal expansion and an inflammatory stimulus to stimulate IL-12 production by macrophages or dendritic cells necessary to induce competency to transcribe the *Ifng* gene. Due to defects in the epigenetic programme played out at the *Ifng* locus, NOD T cells differentiate into IFN-γ-producing effector cells by an autocrine pathway. Thus, NOD T cells of both the $CD4^+$ and $CD8^+$ lineages are driven to differentiate into IFN-γ-producers by IL-2, which T cells produce, as well as by IL-12, which monocyte-lineage cells produce in response to inflammatory stimuli. Results also suggest that these differences between NOD and other strains are genetic differences rather than a reflection of the diabetic process since they are clearly seen in naive T cells and in the closely related NOR strain that doesn't develop diabetes. These intrinsic differences in NOD T cells may represent a genetic trait that favours development of cell-mediated autoimmune diseases such as type 1 diabetes. Thus, epigenetics at the *Ifng* locus, which may be controlled in part by the underlying genetic code, play key roles in strain- and lineage-dependent regulation

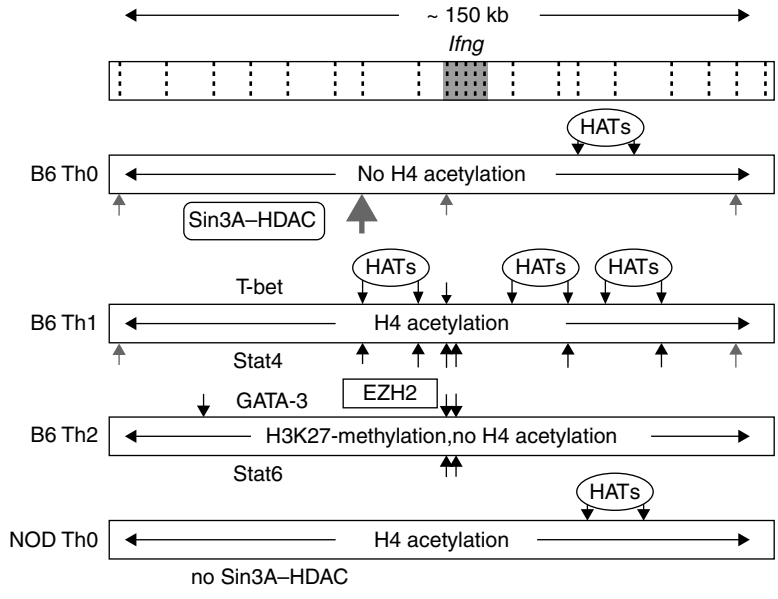

Figure 14.1 Epigenetic regulation of *Ifng* transcription. The *Ifng* gene spans approximately 5 kb of genomic DNA (grey box). Numerous evolutionarily conserved sequences (>70% sequence conservation between rodents and humans over a distance of >100 bp, dashed lines) are positioned 5′ and 3′ of the *Ifng* gene. Sin3A–HDAC complexes dominate the locus in proliferating C57BL/6 T cells, explaining the absence of long-range histone acetylation (grey arrows; Sin3A–HDAC complexes are heavily recruited to the CNS site at −22 kb from the *Ifng* start site). T-bet and STAT6 are recruited to multiple evolutionarily conserved regions in C57BL/6 Th1 cells (black arrows). Most of the HDAC complexes are lost and replaced by HAT complexes. GATA-3 and STAT6 are recruited to the *Ifng* locus in Th2 cells and the histone H3K27 methyltransferase, EZH2, is recruited to the *Ifng* gene by GATA-3-catalysing long-range H3K27 methylation leading to gene repression. In proliferating NOD T cells (NOD Th0), Sin3A–HDAC complexes are not recruited to the *Ifng* locus thus favouring long-range H4 acetylation and gene transcription. Adapted from [15,16,44].

of transcription, corresponding cell-fate decisions, and possibly susceptibility to diabetes (Figure 14.1).

Defects in the DNA-binding proteins required to recruit Sin3–HDAC complexes to the *Ifng* locus could produce more generalized effects on transcriptional regulation. In this scenario, these DNA-binding proteins may be necessary to produce hypo-acetylated domains at a variety of genes and repress their transcription. Additionally, many DNA-binding proteins, such as the transcription factors nuclear factor κB or YY-1, recruit either HATs or HDACs to individual DNA elements in a context-dependent manner. Thus, lack of or defects in transcription factors such as these may stimulate expression of genes that should be repressed and inhibit expression of genes that should be expressed in a given cell type, resulting in a more widespread effect on transcriptional profiles. This is somewhat reminiscent of what is seen in human T cells from patients with SLE. Treatment of cells with a general HDAC inhibitor corrects

expression profiles of certain genes to the control configuration, resulting in increases in expression of the *Ifng* gene and decreases in expression of the *Il10* and *CD154* genes [30].

A third possibility is that NOD T cells have a defective Sin3A–HDAC complex that is not efficiently recruited to DNA. This complex can contain multiple HDACs, Sin3A, the Sin3A-associated proteins, SAP30 and SAP18, and other associated proteins such as RbAp46/48. Lack of function or reduced expression of any of these component parts could lead to reduced ability of these complexes to be recruited to target DNA, resulting in excessive histone hyperacetylation. Such defects could have wide-ranging effects on gene-expression profiles. Nevertheless, focusing on differences in how the histone code is established across the *Ifng* locus in T cells from NOD mice that develop autoimmune disease and in T cells from C57BL/6 mice that do not develop autoimmune disease should address these different possibilities and increase our understanding of how defects in establishing long-range histone acetylation patterns across the *Ifng* locus contribute to autoimmunity.

14.7 Perspectives

Genome-wide studies clearly demonstrate that epigenetic modifications, including activating and repressive histone marks, are most frequently localized to promoters of genes [17,18]. Gene loci that exhibit long-range epigenetic modifications are typically genes that are members of gene families (e.g. the growth hormone gene locus), genes that exhibit developmental alterations in transcription patterns (e.g. the globin gene locus), genes that exhibit lineage-specific expression patterns (e.g. the *Ifng* gene), genes that exhibit stimulus-dependent transcription (e.g. the *Ifng* gene) or genes that require distal DNA elements for their transcription (e.g. the *Ifng* gene). However, the above properties are characteristic of many cytokines and chemokines whose function is to orchestrate the innate and adaptive immune responses. In large part, expression of these genes is restricted to cells of the haematopoietic system and is stimulus-dependent, and distal DNA elements play critical roles in their transcription. Therefore, it is reasonable to speculate that many genes critical to the immune system exhibit complex epigenetic patterns including the presence of long-range histone activating and repressive marks. Since cytokines and chemokines are such potent effector proteins, it is easy to see how their expression needs to be under very strict control. IFN-γ is critically important in controlling both the adaptive and innate arms of the immune system. Under-expression of *Ifng* would reduce the ability of the immune system to control pathogen replication and invasion, leading to bacteraemia, viraemia or even death. Over-expression of *Ifng* could lead to cytokine-mediated inflammation, high morbidity and even mortality. As described here, failures in the long-range epigenetic regulation of transcription of *Ifng* and other cytokine genes could also contribute to autoimmunity by producing too much or too little of key cytokines or chemokines in peripheral tissues.

14.8 Acknowledgements

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References

1. van Holde, K.E. (1988) *Chromatin*. Springer Verlag, New York.
2. Li, B., Carey, M. and Workman, J.L. (2007) The role of chromatin during transcription. *Cell* **128**, 707–719.
3. Berger, S.L. (2002) Histone modifications in transcriptional regulation. *Current Opinions in Genetics and Development* **12**, 142–148.
4. Strahl, B.D. and Allis, C.D. (2000) The language of covalent histone modifications. *Nature* **403**, 41–45.
5. Margueron, R., Trojer, P. and Reinberg, D. (2005) The key to development: interpreting the histone code? *Current Opinions in Genetics and Development* **15**, 163–176.
6. Turner, B.M. (2002) Cellular memory and the histone code. *Cell* **111**, 285–291.
7. Berger, S.L. (2007) The complex language of chromatin regulation during transcription. *Nature* **447**, 407–412.
8. Bottomley, M.J. (2004) Structures of protein domains that create or recognize histone modifications. *EMBO Reports* **5**, 464–469.
9. Metzger, E., Wissmann, M., Yin, N. *et al.* (2005) LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. *Nature* **437**, 436–439.
10. Trojer, P. and Reinberg, D. (2006) Histone lysine demethylases and their impact on epigenetics. *Cell* **125**, 213–217.
11. Bernstein, B.E., Mikkelsen, T.S., Xie, X. *et al.* (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* **125**, 315–326.
12. Dutnall, R.N. (2003) Cracking the histone code: one, two, three methyls, you're out! *Molecular Cell* **12**, 3–4.
13. Forsberg, E.C. and Bresnick, E.H. (2001) Histone acetylation beyond promoters: long-range acetylation patterns in the chromatin world. *Bioessays* **23**, 820–830.
14. Forsberg, E.C., Downs, K.M. and Christensen, H.M. (2000) Developmentally dynamic histone acetylation pattern of a tissue-specific chromatin domain. *Proceedings of the National Academy of Sciences USA* **97**, 14494–14499.
15. Zhou, W., Chang, S. and Aune, T.M. (2004) Long-range histone acetylation of the Ifng gene is an essential feature of T cell differentiation. *Proceedings of the National Academy of Sciences USA* **101**, 2440–2445.
16. Chang, S. and Aune, T.M. (2005) Histone hyperacetylated domains across the Ifng gene region in natural killer cells and T cells. *Proceedings of the National Academy of Sciences USA* **102**, 17095–17100.
17. Bulger, M. (2005) Hyperacetylated chromatin domains: lessons from heterochromatin. *Journal of Biological Chemistry* **280**, 21689–21692.
18. Calestagne-Morelli, A. and Ausio, J. (2006) Long-range histone acetylation: biological significance, structural implications, and mechanisms. *Biochemistry and Cell Biology* **84**, 518–527.
19. Su, R.C., Brown, K.E., Saaber, S. *et al.* (2004) Dynamic assembly of silent chromatin during thymocyte maturation. *Nature Genetics* **36**, 502–506.

20. Kimura, A.P., Liebhaber, S.A. and Cooke, N.E. (2004) Epigenetic modifications at the human growth hormone locus predict distinct roles for histone acetylation and methylation in placental gene activation. *Molecular Endocrinology* **18**, 1018–1032.
21. Avni, O., Lee, D. Macian, F. *et al.* (2002) T(H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. *Nature Immunology* **3**, 643–651.
22. Felsenstein, J. and Churchill, G.A. (1996) A Hidden Markov Model approach to variation among sites in rate of evolution. *Molecular Biology and Evolution* **13**, 93–104.
23. Siepel, A., Bejerano, G., Pedersen, J.S. *et al.* (2005) Evolutionarily conserved elements in vertebrate, insect, worm, and yeast genomes. *Genome Research* **15**, 1034–1050.
24. Van Speybroeck, L. (2002) From epigenesis to epigenetics: the case of C. H. Waddington. *Annals of the New York Academy of Sciences* **981**, 61–81.
25. Feinberg, A.P. (2007) Phenotypic plasticity and the epigenetics of human disease. *Nature* **447**, 433–440.
26. Feinberg, A.P. (2004) The epigenetics of cancer etiology. *Seminars in Cancer Biology* **14**, 427–432.
27. Xu, G.L., Bestor, T.H., Bourc'his, D. *et al.* (1999) Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. *Nature* **402**, 187–191.
28. Richardson, B. (2003) DNA methylation and autoimmune disease. *Clinical Immunology* **109**, 72–79.
29. Forster, N., Gallinat, S. Jablonska, J. *et al.* (2007) p300 protein acetyltransferase activity suppresses systemic lupus erythematosus-like autoimmune disease in mice. *Journal of Immunology* **178**, 6941–6948.
30. Mishra, N., Brown, D.R., Olorenshaw, I.M. and Kammer, G.M. (2001) Trichostatin A reverses skewed expression of CD154, interleukin-10, and interferon-gamma gene and protein expression in lupus T cells. *Proceedings of the National Academy of Sciences USA* **98**, 2628–2633.
31. Mishra, N., Reilly, C.M. Brown, D.R. *et al.* (2003) Histone deacetylase inhibitors modulate renal disease in the MRL-lpr/lpr mouse. *Journal of Clinical Investigation* **111**, 539–552.
32. Garcia, B.A., Busby, S.A. Shabanowitz, J. *et al.* (2005) Resetting the epigenetic histone code in the MRL-lpr/lpr mouse model of lupus by histone deacetylase inhibition. *Journal of Proteome Research* **4**, 2032–2042.
33. Das, P.M., Ramachandran, K., vanWert, J. and Singal, R. (2004) Chromatin immunoprecipitation assay. *Biotechniques* **37**, 961–969.
34. Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual Reviews of Immunology* **7**, 145–173.
35. Harrington, L.E., Hatton, R.D. Mangan, P.R. *et al.* (2005) Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nature Immunology* **6**, 1123–1132.
36. Grogan, J.L., Mohrs, M. Harmon, B. *et al.* (2001) Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. *Immunity* **14**, 205–215.
37. Ansel, K.M., Lee, D.U. and Rao, A. (2003) An epigenetic view of helper T cell differentiation. *Nature Immunology* **4**, 616–623.
38. Lee, G.R., Kim, S.T. Spilianakis, C.G. *et al.* (2006) T helper cell differentiation: regulation by cis elements and epigenetics. *Immunity* **24**, 369–379.
39. Szabo, S.J., Sullivan, B.M., Peng, S.L. and Glimcher, L.H. (2003) Molecular mechanisms regulating Th1 immune responses. *Annual Reviews of Immunology* **21**, 713–758.
40. Wilson, C.B. and Merkenschlager, M. (2006) Chromatin structure and gene regulation in T cell development and function. *Current Opinion in Immunology* **18**, 143–151.
41. Baguet, A. and Bix, M. (2004) Chromatin landscape dynamics of the Il4-Il13 locus during T helper 1 and 2 development. *Proceedings of the National Academy of Sciences, USA* **101**, 11410–11415.

42. Schoenborn, J.R., Dorschner, M.O., Sekimata, M. *et al.* (2007) Comprehensive epigenetic profiling identifies multiple distal regulatory elements directing transcription of the gene encoding interferon-gamma. *Nature Immunology* **8**, 732–742.
43. Eivazova, E.R. and Aune, T.M. (2004) Dynamic alterations in the conformation of the Ifng gene region during T helper cell differentiation. *Proceedings of the National Academy of Sciences, USA* **101**, 251–256.
44. Chang, S. and Aune, T.M. (2007) Dynamic changes in histone-methylation 'marks' across the locus encoding interferon-gamma during the differentiation of T helper type 2 cells. *Nature Immunology* **8**, 723–731.
45. Heinzel, T., Lavinsky, R.M., Mullen, T.M. *et al.* (1997) A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature* **387**, 43–48.
46. Nagy, L., Kao, H.Y., Chakravarti, D. *et al.* (1997) Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* **89**, 373–380.
47. Atkinson, M.A. and Leiter, E.H. (1999) The NOD mouse model of type 1 diabetes: as good as it gets? *Nature Medicine* **5**, 601–604.
48. Castano, L. and Eisenbarth, G.S. (1990) Type-I diabetes: a chronic autoimmune disease of human, mouse, and rat. *Annual Reviews of Immunology* **8**, 647–679.
49. Zhou, W., Zhang, F. and Aune, T.M. (2003) Either IL-2 or IL-12 is sufficient to direct Th1 differentiation by nonobese diabetic T cells. *Journal of Immunology* **170**, 735–740.

Abstract

The histone code hypothesis proposes that cell-fate decisions are achieved through creation of stable epigenetic histone marks at gene loci. These marks can be localized to promoters and transcribed regions of genes or can extend many kilobases beyond these boundaries. The *Ifng* gene exhibits complex activating and repressive patterns of epigenetic modifications that cover a region spanning over 50 kb of upstream and downstream genomic DNA in cells that express or silence *Ifng*. Failure to properly establish this long-range histone code may contribute to the characteristic overproduction of interferon (IFN)-γ by proliferating T cells from mice that develop autoimmune diabetes.

Key words: Th1; Th2; epigenetics; histone code hypothesis; differentiation; histone acetyltransferase; histone deacetylase; evolutionarily conserved DNA sequence; NOD/ShiLtJ mice; C57BL/6J mice; autoimmunity

15

Roquin defects reveal a role for the microRNA machinery in regulating autoimmunity

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15.1 Introduction

The immune system faces the constant challenge of maintaining tolerance to normal self-components of the body while mounting robust responses to invading pathogens. Defects in immunological tolerance can lead to a wide range of systemic and organ-specific autoimmune diseases. As the cellular and molecular mechanisms responsible for maintaining tolerance are being deciphered, it is becoming clear that spatiotemporal control of gene expression underpins most successful tolerance mechanisms. Transcriptional control and chromatin modification are major determinants of gene expression, which is fine-tuned post-transcriptionally to enable cells to respond rapidly to changes in intracellular and extracellular stimuli, immediately before protein synthesis. In particular, small non-coding RNAs that bind to specific sequences in the untranslated regions (UTRs) of their target transcripts have been shown to play essential roles in regulating mRNA stability and translation [1]. Among small non-coding RNAs, microRNAs (miRNAs) have been shown to play a major role in development, acting on cellular differentiation and organismal diversification. This level of post-transcriptional regulation has attracted recent attention due to accumulating evidence that its malfunction may lead to neoplastic cellular transformation and several human diseases [2].

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15.2 RNA silencing through the miRNA machinery

miRNAs were first discovered 15 years ago as regulators of developmental timing in *Caenorhabditis elegans* [3,4]. Since then, RNA silencing through miRNAs has been shown to be crucial for multiple aspects of plant and animal development. miRNAs constitute a class of small non-coding RNAs of 20–22 nucleotides in length that bind to specific sequences within the 3′ and/or 5′ UTRs of target mRNAs and mediate post-transcriptional repression of gene expression. They are often encoded within the introns of protein-coding genes, but can also be found as independent transcription units or in polycystronic clusters [5]. After being transcribed by RNA polymerase II as primary RNAs (pri-miRNA) with a long hairpin structure, they are generally cleaved in the nucleus by the RNase III enzyme Drosha. This yields the approximately 65-nucleotide precursor miRNA (pre-miRNA) containing the hairpin structure, which is exported to the cytoplasm for further processing by a different RNase III enzyme, Dicer (Figure 15.1). The result is a 19–25 double-stranded duplex; the mature miRNA, which is retained into the functional miRNA-induced silencing complex (miRISC). miRISC also contains an Argonaute protein and other protein co-factors. The miRISC can then bind to mRNAs that have sequences complementary to the miRNA. This complementarity tends to be imperfect in animals, except for a region between

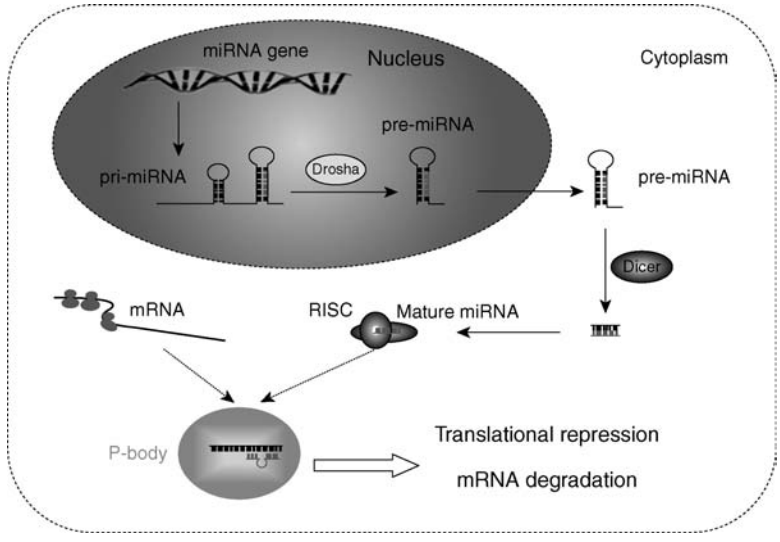

Figure 15.1 Mammalian miRNA biogenesis and function. In the mammalian genome, miRNA genes encode for a long primary miRNA transcript (pri-miRNA). This is processed into a precursor miRNA (pre-miRNA) by the nuclear RNase III enzyme Drosha. After being actively transported to the cytoplasm, the pre-miRNA is further processed by the RNase III enzyme Dicer. The RNA-induced silencing complex (RISC) recognizes the miRNA duplex and loads the functional strand of the duplex (mature miRNAs) on to the target RNA in an imperfectly complementary manner to repress its translation or induce degradation in processing (P) bodies.

residues 2 and 8 at the 5′ end of the miRNA known as the seed region, which is highly complementary [6,7].

Inhibition of target mRNAs generally occurs through translational inhibition or mRNA degradation [8] (Figure 15.1). In cases of perfect complementarity between miRNA and target, as tends to occur in plants, target mRNA cleavage and degradation occurs. Several hundred miRNAs are predicted to regulate about 30% of mammalian protein-encoding mRNAs [7]. While some miRNAs can target over 100 genes, there also appears to be some functional redundancy among them. Although the genes targeted by a single miRNA do not necessarily share functions nor need to belong to the same biological pathway, there are numerous examples to suggest that miRNAs can regulate pathways by coordinately repressing multiple targets with related functions [7].

15.3 miRNAs regulate lymphoid cell development and immune responses

Immune competence depends on differentiation of B, T and myeloid cells into functional subsets, compartmentalization of cell subsets within microenvironments in secondary lymphoid tissue, and lymphocyte homeostasis. Each of these processes is controlled, at least in part, by specific miRNAs. A cohort of recent papers using miRNA expression profiling has revealed striking differences in the expression of highly conserved miRNAs by specific haematopoietic lineages, and during different developmental stages [9–12]. A good example of an miRNA regulating lymphoid cell differentiation is miR-181. Mature miR-181 expression levels are gradually upregulated through the transition between undifferentiated progenitor cells to differentiated B lymphocytes. Ectopic expression of miR-181a in haematopoietic stem cells enhances the differentiation of $CD19^+$ B cells and suppresses the differentiation of $CD8^+$ T cells [9]. For myeloid cells, miR-223 has been shown to be a myeloid-specific miRNA and appears to be driven by the myeloid transcription factors PU.1 and CCAAT/enhancer-binding protein (C/EBP). Ectopic expression of miR-223 in an acute promyelocytic leukaemia cell line resulted in the upregulation of the myelomonocytic differentiation markers CD11b and CD14, and granulocyte colony-stimulating factor receptor, as well as inducing morphological changes consistent with cell maturation [13,14].

miRNAs are also emerging as critical sensors and regulators of immune responses playing key roles in setting the threshold of lymphoid and myeloid cell activation in response to stimulation of antigen receptors, Toll-like receptors (TLRs) and cytokine receptors. Elegant examples of this level of regulation include the recently described roles of miR-181 and miR-146. miR-181a is highly expressed in immature T cells and reduces the threshold of T cell receptor (TCR) signalling, facilitating both positive and negative selection. This is achieved through dampening the expression of multiple phosphatases that are negative regulators of distinct steps of the TCR signalling cascade, such as SH2-domain-containing protein tyrosine phosphatase 2 (SHP2), protein

tyrosine phosphatase, non-receptor type 22 (PTPN22), dual-specificity protein phosphatase 5 (DUSP5) and DUSP6 [15].

Bacterial components can also induce expression of miRNAs. In response to lipopolysaccharide (LPS) and pro-inflammatory cytokines including interleukin (IL)-1β and tumour necrosis factor α (TNF-α), expression of miR-146 expression is upregulated in human monocytes. Importantly, miR-146 represses the expression of TNF receptor-associated factor 6 and IL-1 receptor-associated kinase 1, the key adaptor molecules in TLR and IL-1 receptor signalling cascades [16]. In doing this, miR-146 negatively controls TLR and cytokine receptor signalling. This is a powerful example of how miRNAs can also be instrumental in establishing negative-feedback loops to terminate pro-inflammatory signals.

miRNAs are also involved in RNA interference (RNAi)-mediated immune defence against viruses. RNAi can be mediated by small silencing RNAs, such as short interfering RNAs (designed to be perfectly complementary to a region of the target mRNA sequence) and by miRNAs (that are in general imperfectly complementary to a region of the target mRNA sequence) [8]. The role of RNAi as a first-line tool of antiviral defence in plants and invertebrates has been well supported by many lines of evidence, particularly those demonstrating that the RNA-silencing mechanism protects hosts against viral infections and that essential virulence factors of many viruses are viral suppressors of RNA silencing [17].

In mammals, viral miRNAs can control expression of cellular genes to interfere with antiviral host defence. Hcmv-miR-UL112 expressed by human cytomegalovirus has been shown to protect infected cells from the killing by natural killer (NK) cells by repressing expression of the NK cell-activating ligand, major histocompatibility complex (MHC) class I-related chain B (MICB) [18]. Cellular miRNAs can also act to suppress viral infection. For example, cellular miRNAs miR-24 and miR-93 target the RNA genome of the rhabdovirus vesicular stomatitis virus (VSV) [19]. Due to the low fidelity of viral RNA-dependent RNA polymerases, it would be very risky to solely rely on sequence complementarity to defend against viral infections. Indeed, other defence mechanisms, including sensing pathogen-specific patterns by TLRs, have evolved in mammals, and are complementary to miRNA-mediated defence [20].

15.4 miRNAs as single drivers of immunodeficiency or inflammation

Considering the broad and diverse functions of miRNAs in the immune system, and their power to regulate hundreds of target genes with related functions, it is not surprising to observe phenotypes of both under- or overactive immune responses in animals deficient in a single miRNA. The first miRNA-knockout mouse showing a distinct immunological phenotype was the miR-155-deficient strain. *miR-155*$^{-/-}$ mice showed defects in B cell, T cell and dendritic cell function and displayed impaired helper T cell differentiation and decreased T cell-dependent antibody production and affinity maturation [21–23]. Deficiency in *miR-155*$^{-/-}$ mice also led to failure to mount

protective responses against *Salmonella* infection [21]. This phenotype is explained at least in part by the demonstration that the T helper (Th) 2 cytokine-enhancing transcription factor c-Maf and B cell terminal differentiation-related transcription factor PU.1 are physiological targets of miR-155. Other target candidates include several cytokines and chemokines [21,23].

miRNA deficiency does not only cause immunodeficiency; importantly, selective miRNA deficiencies can also lead to overactive immune phenotypes and enhanced inflammatory responses. Two examples of the latter include mice deficient in miR-150 or miR-223. miR-150 is a mature lymphocyte-specific miRNA that targets the transcription factor c-Myb and deficiency of this miRNA in mice has shown to lead to B1 cell expansion and an enhanced humoral immune response [24]. Studies on *miR-223*$^{-/-}$ mice have revealed that this myeloid-specific miRNA controls granulocyte maturation and activation by targeting myeloid progenitor proliferation-enhancing transcription factor Mef2c. Neutrophil hyperactivity seen in *miR-223*$^{-/-}$ mice leads to spontaneous development of inflammatory lung pathology and exaggerated tissue destruction after endotoxin challenge [25].

15.5 miRNAs regulate autoimmunity

The trade off with having the capability to mount potent immune responses is the risk of autoimmunity. Therefore, the delicate balance between protective and autoimmune responses may hinge on miRNA-mediated fine-tuning of gene expression. Indeed, recent reports suggest that miRNAs may play important roles in repressing immune responses against self. Systemic lupus erythematosus (SLE, or lupus) was the first autoimmune disease shown to be regulated by the miRNA machinery. Lupus is the prototypic systemic autoimmune disease with a female to male ratio of 9:1, affecting approximately 1 in 700 women of childbearing age. The clinical manifestations are diverse, and include malaise, lymphoid organ enlargement, skin rashes, lymphopenias and kidney failure, among others. At the core of the pathogenesis is the aberrant production of autoantibodies against nuclear antigens, including anti-double-stranded DNA (dsDNA) antibodies, which are pathogenic. The lupus-associated repertoire of self-antigens targeted by autoantibodies is normally intracellular, although certain nuclear antigens become exposed on the cell surface during apoptosis.

Lupus-susceptibility alleles identified in genome-wide association studies in patients and mouse models include genes that affect T and B cell activation thresholds, especially to nucleic acids and ribonuclear proteins, antigen presentation, plasmacytoid dendritic cell activation and interferon (IFN)-α production, myeloid cell migration and recruitment, components of the complement cascade, and propensity to apoptosis. Molecular defects that compromise the numerous mechanisms that normally affect efficient and non-immunogenic clearance of apoptotic cells have also been implicated in lupus pathogenesis. Thus, the heterogeneity of clinical lupus is mirrored in the diversity of pathophysiological pathways. It is of particular importance that lupus phenotypes are often the result of dose-dependent outcome of changes in key regulators

of the immune response, rather than absolute deficiencies. Two recent studies identifying a link between defects in miRNA-mediated RNA silencing and development of lupus have emphasized the physiological relevance of post-transcriptional regulation of proteins that, as highlighted below, function over a tight range of concentrations during normal immune responses.

The first of these studies describing a role for the mRNA machinery in preventing systemic autoimmunity utilized a strain of mice homozygous for a hypomorphic variant of the *roquin* gene (*sanroque* mice), which develop lupus and autoimmune diabetes [26]. Lymphoproliferation in these mice is caused by over-expression of the inducible T cell co-stimulator (ICOS), due to decreased miR-101-mediated ICOS mRNA decay [27]. Subsequent to this report, over-expression of the miRNA 17–92 cluster, frequently amplified in lymphoma, has also been found to drive lymphoproliferation and a lupus-like disease through failure to downregulate *Pten* and *Bim* transcripts, two important negative regulators of T cell activation, proliferation and survival [28]. In the remainder of this chapter, we will describe in some detail how Roquin regulates miRNA-mediated post-transcriptional regulation to prevent autoimmune manifestations and how this knowledge can be used to explore novel therapeutic avenues.

15.6 Roquin regulates miRNA-mediated silencing of T cells and represses lupus

The *sanroque* strain was generated in an effort to illuminate autoimmune regulators through controlled variation of the mouse genome with the chemical mutagen, *N*-ethyl-*N*-nitrosourea, combined with a set of sensitive immunological screens [29]. *Sanroque* female mice develop anti-nuclear antibodies by 6–7 weeks of age whereas these autoantibodies can only be detected in male mice after 8 weeks of age [30]. Anti-nuclear antibodies are also detected earlier in female SLE patients [31]. Mice homozygous for the *san* allele of *roquin* develop many other typical SLE features, including high-affinity antibodies against dsDNA, focal proliferative glomerulonephritis with deposition of IgGs-containing immune complexes, anaemia and autoimmune thrombocytopenia as well as other autoimmune manifestations such as lymphadenopathy, splenomegaly, necrotizing hepatitis and plasmacytosis in lymph node medullary cords, kidney, liver and lung [30]. *Sanroque* mice are also susceptible to other autoimmune diseases. When crossed with a diabetes-susceptible genetic background in which intact T cell tolerance mechanisms normally prevent islet cell destruction (TCR^{HEL}:insHEL double transgenic mice [32]), 100% of *sanroque* mice develop diabetes as early as 4 weeks of age [30]. The mutation causing the autoimmune phenotype was mapped to a new gene, *roquin*, with previously unknown function. *Roquin* (*Rc3h1*) encodes a member of the RING-type E3 ubiquitin ligase protein family. *Sanroque* mice carry a T → G substitution in *roquin* (*san* allele), resulting in a non-conservative Met-199 → Arg codon change in a novel protein domain (ROQ domain) with high conservation from *C. elegans* to humans (Figure 15.2 and see below) [30].

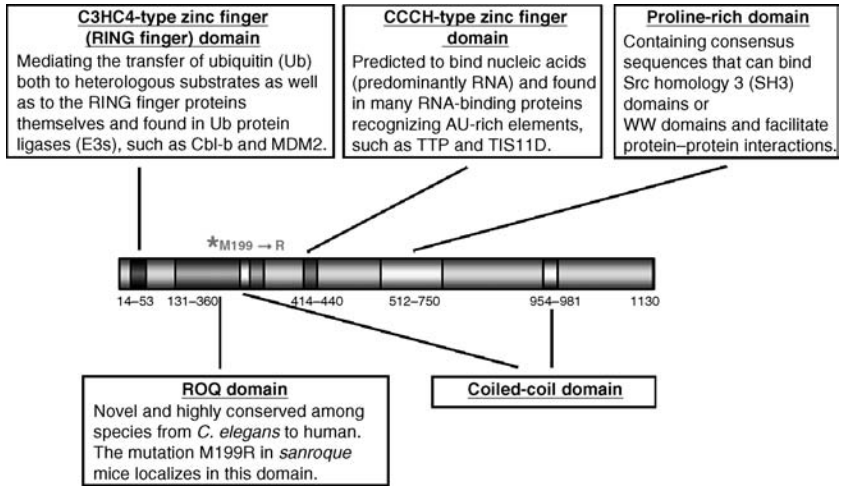

Figure 15.2 Representative structure of Roquin protein. Sequence homology suggests Roquin contains domains with putative ubiquitin protein ligase and nucleic acid-binding functions. Numbers indicate amino acid positions. M199 → R, Met-199 → Arg mutation. C3HC4, a Cys × 3-His-Cys × 4-type zinc finger (RING finger) domain; CCCH, a Cys-Cys-Cys-His (CCCH)-type zinc finger domain.

Dysregulated follicular helper T cells and germinal centre responses lead to lupus

Roquin^{M199R} causes hyperactivation of CD4$^+$ T cells. By 8 weeks of age, approximately 50% of CD4$^+$ T cells in *sanroque* mice show an activated/memory phenotype with high expression levels of CD44, compared with approximately 20% in wild-type mice. One of the most striking phenotypic features of *sanroque* T cells is the consistent over-expression of inducible ICOS on both naïve (CD44^{low}) and activated/memory (CD44^{high}) CD4$^+$ T cell subsets. CD4$^+$ T cell hyperactivation and aberrant expression of ICOS was shown to be T cell autonomous: in mice whose haematopoietic system was reconstituted with cells containing a 50%/50% mix of *sanroque* and wild-type bone marrow cells, only CD4$^+$ T cells derived from *sanroque* bone marrow displayed a high percentage of CD44^{high} cells and expressed higher levels of ICOS [30]. Large spontaneous germinal centres formed in *sanroque* mice as young as 6–8 weeks, accompanied by a large amount of T cells located within germinal centres [30]. Gene-expression profiling revealed that CD4$^+$ T cells from *sanroque* mice differentially upregulated the expression of genes such as *Icos*, *Cxcr5*, *Cd200*, *Cd84*, *Bcl6*, *Il21*, *neuropilin 1* and *Ccl5*, all of which are markers characteristic of follicular helper T (T_{FH}) cells. T_{FH} cells constitute a specialized helper T cell subset that supports germinal-centre reactions and selects germinal-centre B cells to differentiate into high-affinity memory cells or long-lived plasma cells [26,33–35].

It has been long known that most anti-dsDNA antibodies detected in humans and in animal models of SLE are high-affinity IgGs antibodies, which suggests they might be generated in germinal-centre reactions [36]. The process of somatic hypermutation

typically occurs in the germinal centre, and targets the immunoglobulin variable-region genes of rapidly dividing germinal-centre B cells (centroblasts). This can lead to an increase in the affinity of the B cell receptor for the immunizing antigen, but it can also lead to self-reactive specificities. Thus, a tightly controlled process of germinal-centre B cell selection by antigen-specific T_{FH} cells is normally in place to ensure positive selection of those cells with the highest affinity towards foreign antigens while preventing selection of cells that have become self-reactive. Mutated germinal-centre B cells that fail to receive pro-survival signals are programmed to die by apoptosis, providing a mechanism that prevents the production of high-affinity, long-lived self-reactive B cells [37]. Other mechanisms, including exclusion of self-reactive B cells from entering germinal centres, also contribute to prevent formation of high-affinity autoantibodies, and have been shown to fail in SLE patients [38]. The randomness of the mutation process, together with the longevity of post-germinal-centre memory B cells and plasma cells, and the abundance of exposed nuclear antigens on the surface of numerous germinal-centre cells undergoing apoptosis, rank germinal-centre reactions highest in the risk of triggering and maintaining autoantibody-driven autoimmunity.

The abnormal accumulation of T_{FH} cells and spontaneous formation of germinal centres in unimmunized *sanroque* mice suggest that autoantibodies including anti-nuclear antibodies may be the product of T cell-dependent B cell activation and differentiation in germinal centres, driven by self-antigen. It also suggests that germinal-centre exclusion of self-reactive T_{FH} cells or limiting T_{FH} cell-derived helper signals to germinal-centre B cells is required to prevent self-reactivity arising in follicles.

Over-expression of ICOS contributes to sanroque autoimmunity

Four important mechanisms have been described that are essential to maintain T cell tolerance and prevent autoimmunity: (1) deletion of self-reactive T cells in the thymus, which is critically dependent on autoimmune regulator (AIRE)-mediated expression of organ-specific antigens by thymic epithelial cells [39,40], intact TCR signalling through ZAP70 [41] and intact Bcl-2-interacting mediator (BIM)-mediated TCR-induced thymocyte death [37]; (2) co-stimulation-dependent T cell activation maintained by the E3 ubiquitin ligase Cbl-b [42,43], and terminated by the inhibitory co-receptor cytotoxic T lymphocyte antigen 4 (CTLA-4) [44,45]; (3) T cell activation-induced cell death (AICD) mediated by the pro-apoptotic receptor Fas and its ligand, FasL [46], and (4) regulatory T cell (Treg)-mediated repression, dependent on developmentally regulated expression of the Treg-specific transcription factor Foxp3 on a subset of thymocytes [47] and regulation of Treg development and homeostasis by the cytokine IL-2 and its receptor [48]. None of the above T cell tolerance mechanisms is impaired in *sanroque* mice, which demonstrate normal self-reactive T cell deletion in the thymus, Treg cell-mediated repression, CD28 co-stimulation-dependent T cell proliferation, FasL-dependent activation-induced T cell death and normal induction of the critical B

cell helper molecule CD40L [30]. Instead, aberrantly over-expressed ICOS on T cells in *sanroque* mice emerged as the key culprit for the break in self-tolerance.

The *Icos* gene is a paralogue of the evolutionarily more ancient co-stimulator *Cd28* [49]. Signals through both CD28 and ICOS can induce T cell activation, differentiation and cytokine production [50]. Nevertheless, CD28 and ICOS act at separate stages of immune responses: CD28 is essential at the stage of priming naïve T cells and ICOS is critical for provision of help to B cells in germinal centres. The defects of mice and humans lacking ICOS or ICOS ligand (ICOSL) demonstrate the important functions of ICOS for production of B cell memory [51–57]. Deficiency in ICOS has been found in several patients with adult-onset common variable immunodeficiency, a disease with a clinical phenotype of low serum immunoglobulin concentrations, defective specific antibody production and increased susceptibility to bacterial infections of the respiratory and gastrointestinal tracts [57].

Recent findings have identified Roquin as the critical switch that maintains functional compartmentalization of CD28 and ICOS to allow their respective key roles in T cell discrimination between pathogens and self and in the provision of T cell selection signals to germinal centre B cells. In the absence of CD28, ICOS over-expression due to defective Roquin can functionally substitute for three important functions of CD28 that are normally selectively dependent on CD28 signalling: (1) T cell priming for generation of T cell-dependent antibody responses, (2) formation of germinal centres and T_{FH} cells and (3) homeostasis of peripheral Tregs (M. Linterman *et al.*, unpublished results). This compartmentalization explains how these duplicated paralogous genes have resolved an adaptive conflict (maintenance of immunological tolerance) while acquiring specialized functions that confer a selective advantage: the appearance of ICOS-enabled birds and mammals to undergo affinity-maturation and formation of memory cells (independently of danger signals), both key for protection against rapidly dividing bacteria, toxins and many viruses. These observations indicate that Roquin has been co-opted in evolution to prevent crosstalk between these two pathways.

Unlike the well-established causal effect of ICOS deficiency on common variable immunodeficiency, a possible role of ICOS over-expression leading to autoimmunity was until recently only associational: higher expression of ICOS had been detected on T cells from patients with SLE and rheumatoid arthritis compared to healthy controls [58–60]. In *sanroque* mice, however, ICOS expression has been conclusively shown to be causally related to autoimmune lymphoproliferation. When the *Icos* gene dosage was halved by interbreeding *Icos*-knockout mice with *sanroque* mice, $CD4^+$ T cells from *sanroque* mice, losing one allele of *Icos* dramatically reduced ICOS expression, although the levels were still higher than those of wild-type mice. This partial correction of ICOS expression was sufficient to ameliorate the lymphadenopathy, splenomegaly, total T and B cell numbers and T_{FH} and germinal-centre B cell expansion [27]. By contrast, halving the dose of CD28 did not ameliorate the autoimmune syndrome of *sanroque* mice.

A two-signal mechanism regulates T cell responses in secondary lymphoid tissues, whereby TCR engagement by antigen/MHC on an antigen-presenting cell (APC)

only triggers T cell accumulation and effector functions when a second co-stimulatory receptor on the T cell, CD28, is simultaneously engaged by B7 proteins that are induced on the APC upon exposure to microbes [61–64]. ICOS can provide co-stimulation for T cell responses in the absence of CD28 [50,65]. ICOSL, unlike B7.1 and B7.2, is expressed constitutively on many APCs in the absence of microbe components, raising a paradox about how autoimmunity is avoided in the face of this second co-stimulatory system. Maintaining low expression of ICOS on naïve T cells may therefore be critical to prevent the activation of T cells by self-antigens. Indeed, the severity of the lymphadenopathy correlated closely with the levels of ICOS expressed on naïve T cells in *sanroque* mice with either one or two alleles of *Icos* [27]. Taken together, these studies on *sanroque* mice reveal a unique mechanism to prevent autoimmunity by limiting ICOS–ICOSL signalling, which is complementary to the mechanism that controls expression of B7 co-stimulatory ligands for CD28 on APCs.

Roquin limits ICOS mRNA through the miRNA machinery

Aberrant expression of ICOS on *sanroque* $CD4^+$ T cells is T cell-autonomous, suggesting that Roquin functions to regulate ICOS expression either directly or indirectly in a cell-intrinsic fashion, which is reinforced by the observation that ectopic expression of Roquin into $CD4^+$ T cells represses ICOS protein expression [30] as well as its mRNA expression [27]. Notably, ICOS mRNAs from different species including human, rat and mouse all have long 3′ UTRs with several highly conserved segments [27]. The 3′ UTR is not under the same rigid structural constraints as the coding region or the 5′ UTR that has to accommodate the translational machinery. Therefore, conserved segments within 3′ UTRs that form under evolutionary pressure may function to regulate mRNAs. Consistent with this, a distal fragment within the 3′ UTR of ICOS mRNA was shown to be required for ICOS repression by Roquin. Specifically, Roquin destabilizes ICOS mRNA [27]. The conserved 3′ UTR segment containing the *cis*-acting elements for Roquin's repressive action contained a predicted target sequence for miR-101 and miR-103. Overexpression of miR-101 in T cells repressed ICOS mRNA and the repression of ICOS by Roquin was at least partially dependent on miR-101 recognition since a two-nucleotide inversion in the miR-101-recognized sequence within *Icos* 3′ UTR impaired its repression [27]. Repression of ICOS by miR-101 is likely to be involved in the physiological regulation of ICOS expression during T cell differentiation, since there is a striking inverse correlation between miR-101 expression and ICOS expression in human T cells: ICOS levels are lowest on naïve T cells, intermediate on activated T and memory cells and highest on T_{FH} cells [66]. By contrast, the highest levels of miR-101 are found in naïve cells, intermediate levels in memory cells and lowest levels in T_{FH} cells [27].

Roquin therefore emerges as a potential RNA-binding protein involved in miRNA-mediated post-transcription regulation (see discussion below). Other proteins in this

category have been shown to be capable of regulating mRNA stability of multiple targets. An example is the AU-rich element (ARE)-binding protein tristetraprolin, which regulates expression of multiple cytokines including TNF-α [67], granulocyte macrophage colony-stimulating factor [68] and IL-2 [69]. Not surprisingly, another surface receptor important for T cell priming, neuropilin 1, and over-expressed in *sanroque* T cells [30], is also a target of Roquin through the action of miR-101: neuropilin 1 mRNA was also repressed by ectopic expression of either Roquin or miR-101 [27]. Roquin thus appears to play an essential role in miRNA-mediated regulation of what we predict will turn out to be multiple transcripts co-ordinately involved in T cell priming and the maintenance of peripheral T cell tolerance.

How does Roquin regulate miRNA-mediated post-transcriptional regulation?

Clues as to how Roquin interacts with the miRNA machinery and/or the target transcripts, and how the mutation in *sanroque* mice affects this process, can be obtained from Roquin's protein sequence analysis and intracellular localization. Roquin contains several conserved domains (from N- to C-terminal): (1) a Cys×3-His-Cys×4 (C3HC4)-type zinc finger (RING finger) domain, recently shown to act as an E3 ubiquitin protein ligase [70], and found in many E3 ubiquitin protein ligases including c-Cbl; (2) a novel ROQ domain that contains the Met-199 → Arg substitution in *sanroque* mice; (3) a Cys-Cys-Cys-His (CCCH)-type zinc finger domain, predicted to bind nucleic acids (predominantly RNA) and found in a range of RNA-binding proteins, such as tristetraprolin; and (4) a proline-rich domain, containing potential sites for binding Src homology 3 (SH3) domains of interacting proteins [30] (Figure 15.2). The presence of a CCCH-type zinc finger domain suggests that Roquin may directly bind mRNA [71]. The prototypic CCCH zinc finger protein tristetraprolin has been shown to bind to AREs in the 3′ UTRs of *TNF* and mediate its degradation [67]. Ubiquitylating and proteasome activity have been shown to be essential for rapid turnover of mRNAs containing AREs [72]. Furthermore, AUF-1 and the mRNA-stabilizing protein, Hu antigen R (HuR), are degraded through the ubiquitination pathway [73], but the responsible E3 ligase is still unknown. It is therefore possible that Roquin's ubiquitin ligase and putative RNA-binding capacities may link the processes of ubiquitination of RNA-binding proteins and mRNA turnover.

Roquin can be found diffusely distributed in the cytoplasm, and upon stress-induction with arsenite treatment localizes to cytoplasmic aggregates containing T cell intracellular antigen 1 (TIA-1), an RNA-binding protein that is a marker for stress granules [30]. Stress granules are sites where, at times of cellular stress, transcripts are maintained in a form of translational arrest and triaged to exosomes for destruction, to polysomes to be translated, or to processing bodies (P-bodies), for miRNA-mediated translational inhibition or mRNA decay [74]. Interestingly, a recent study demonstrated that T cells also form stress granules to post-transcriptionally regulate the expression of T helper cytokines. Transient translational inhibition of cytokine

transcripts in stress granules occurred after T cell priming, but was relieved after re-stimulation [75]. Both the proliferation of primed T cells and the execution of T cell effector functions (cytokine secretion) need a rapid increase in the endoplasmic reticulum load due to an expanded protein repertoire. Stress granule-mediated uncoupling of the two processes may avoid the toxic side effects of endoplasmic reticulum overload [75]. Roquin's localization to stress granules suggests that during stress-inducing conditions Roquin acts to represses ICOS mRNA. This 'stress' could include T cell priming by dendritic cells in the absence of danger signals. Full-length ICOS mRNAs are also found in stress granules, and importantly, the localization of ICOS mRNA to stress granules is mediated by the 3′ UTR but not by the coding sequence [27], suggesting that localization of both ICOS mRNA and Roquin to stress granules is necessary for the regulation of ICOS expression by Roquin. Since Roquin's CCCH zinc finger domain is predicted to bind nucleic acids (predominantly RNA), it is possible that Roquin captures ICOS mRNA in the cytoplasm through a direct interaction, and recruits it into stress granules along with its own entry into this compartment. An alternative but not mutually exclusive possibility is that Roquin might recognize the ICOS mRNA–miRNA complex, in a way similar to how RISC recognizes mature double-stranded miRNAs [76]. It is also possible that the CCCH zinc finger domain may not mediate sequence-specific binding but non-specific binding to multiple RNAs in stress granules stabilizing and maintaining Roquin's localization in that compartment. The finding that full-length ICOS mRNA localizes into P-bodies suggests that once ICOS mRNA is recruited to stress granules, it is routed to P-bodies for degradation [27]. P-bodies have been observed to physically interact with stress granules. The transient association of stress granules and P-bodies can be promoted by the mRNA decay factors tristetraprolin and BRF1 [77,78]. A role for Roquin in facilitating transient interactions between stress granules and P-bodies in T cells is also possible.

One remaining important question is how the Met-199 → Arg mutation in Roquin impairs repression of ICOS. This substitution lies in the novel ROQ domain and has been predicted to alter the local helical structure, thus probably altering protein conformation [30]. Since it does not alter Roquin's subcellular localization, it is possible the Met-199 → Arg mutation impairs its predicted RNA-binding capacity, its E3 ligase function or the association with interacting proteins.

15.7 Concluding remarks

The mammalian immune system has evolved to safeguard against autoimmunity through a complex series of tolerance checkpoints. Animal models of autoimmune disease have illuminated many of these molecular and/or cellular mechanisms, enabling scientists to sketch the atlas of immune tolerance. The recently developed *sanroque* mouse strain has been an informative model, revealing how the less-well understood subset of helper T cells, T_{FH} cells, are critical to maintain tolerance in germinal centres, through the tightly regulated expression of ICOS (Figure 15.3). Roquin has emerged as a key node in an miRNA pathway that represses key T cell-co-stimulatory molecules.

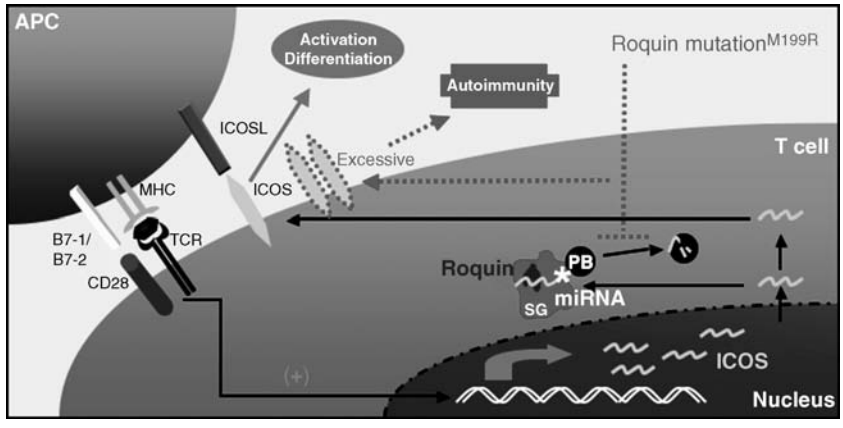

Figure 15.3 Model by which repression of ICOS by Roquin inhibits autoimmunity. In wild-type mice, a significant proportion of *Icos* transcripts induced by TCR and CD28 signalling undergoes degradation and this is enhanced by Roquin acting with the miRNA machinery. The end result is a decrease in the amount of ICOS expressed on the cell surface. After ligation by ICOSL, which is consitutively expressed on APCs, ICOS transduces a signal that provides co-stimulation for T cell activation and differentiation. Limits on ICOS expression imposed by Roquin ensure the maintenance of self-tolerance. In *sanroque* mice, the degradation of *Icos* transcripts is impaired due to the Met-199 → Arg (M199R) mutation in Roquin, resulting in ICOS over-expression on the cell surface. Exaggerated signalling transduced by uncontrolled expression of ICOS contributes to autoimmunity. PB, processing-body; SG, stress granule; miR, microRNA.

At the molecular level, a picture is emerging in which miRNA-mediated gene regulation acts to fine-tune expression of pro-immunogenic molecules whose over- or under-expression may tip the delicate balance between protective immunity and deleterious autoimmunity. At the cellular level, the regulation of the ICOS–ICOSL co-stimulatory axis emerges as a powerful T cell tolerance mechanism complementary to that of controlling expression of the ligands for the main co-stimulator, CD28. At the organism level, a tightly regulated and tolerized T_{FH} population is required to prevent the development of germinal centre-derived autoimmunity. These findings open the way for manipulation of RNA interference to achieve much needed specificity and reduced side effects in the treatment of systemic autoimmunity. They also suggest that polymorphisms in miRNAs and their complementary sequences within untranslated regions of target mRNAs may constitute candidate lupus-susceptibility alleles.

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References

1. Mignone, F., Grillo, G., Licciulli, F. *et al.* (2005) UTRdb and UTRsite: a collection of sequences and regulatory motifs of the untranslated regions of eukaryotic mRNAs. *Nucleic Acids Research* **33**, D141–D146.
2. Conne, B., Stutz, A. and Vassalli, J.D. (2000) The 3' untranslated region of messenger RNA: a molecular 'hotspot' for pathology? *Nature Medicine* **6**, 637–641.
3. Lee, R.C., Feinbaum, R.L. and Ambros, V. (1993) The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75**, 843–854.
4. Wightman, B., Ha, I. and Ruvkun, G. (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell* **75**, 855–862.
5. Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297.
6. Lewis, B.P., Shih, I.H., Jones-Rhoades, M.W. *et al.* (2003) Prediction of mammalian microRNA targets. *Cell* **115**, 787–798.
7. Rajewsky, N. (2006) microRNA target predictions in animals. *Nature Genetics* **38**,(suppl), S8–S13.
8. Jackson, R.J. and Standart, N. (2007) How do microRNAs regulate gene expression? *Science STKE* **2007**, re1.
9. Chen, C.Z., Li, L., Lodish, H.F. and Bartel, D.P. (2004) MicroRNAs modulate hematopoietic lineage differentiation. *Science* **303**, 83–86.
10. Monticelli, S., Ansel, K.M., Xiao, C. *et al.* (2005) MicroRNA profiling of the murine hematopoietic system. *Genome Biology* **6**, R71.
11. Neilson, J.R., Zheng, G.X., Burge, C.B. and Sharp, P.A. (2007) Dynamic regulation of miRNA expression in ordered stages of cellular development. *Genes & Development* **21**, 578–589.
12. Wu, H., Neilson, J.R., Kumar, P. *et al.* (2007) miRNA profiling of naive, effector and memory CD8 T cells. *PLoS ONE* **2**, e1020.
13. Fazi, F., Rosa, A., Fatica, A. *et al.* (2005) A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell* **123**, 819–831.
14. Fukao, T., Fukuda, Y., Kiga, K. *et al.* (2007) An evolutionarily conserved mechanism for microRNA-223 expression revealed by microRNA gene profiling. *Cell* **129**, 617–631.
15. Li, Q.J., Chau, J., Ebert, P.J. *et al.* (2007) miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell* **129**, 147–161.
16. Taganov, K.D., Boldin, M.P., Chang, K.J. and Baltimore, D. (2006) NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proceedings of the National Academy of Sciences USA* **103**, 12481–12486.
17. Li, F. and Ding, S.W. (2006) Virus counterdefense: diverse strategies for evading the RNA-silencing immunity. *Annual Review of Microbiology* **60**, 503–531.
18. Stern-Ginossar, N., Elefant, N., Zimmermann, A. *et al.* (2007) Host immune system gene targeting by a viral miRNA. *Science* **317**, 376–381.
19. Otsuka, M., Jing, Q., Georgel, P. *et al.* (2007) Hypersusceptibility to vesicular stomatitis virus infection in Dicer1-deficient mice is due to impaired miR24 and miR93 expression. *Immunity* **27**, 123–134.
20. Muller, S. and Imler, J.L. (2007) Dicing with viruses: microRNAs as antiviral factors. *Immunity* **27**, 1–3.
21. Rodriguez, A., Vigorito, E., Clare, S. *et al.* (2007) Requirement of bic/microRNA-155 for normal immune function. *Science* **316**, 608–611.
22. Thai, T.H., Calado, D.P., Casola, S. *et al.* (2007) Regulation of the germinal center response by microRNA-155. *Science* **316**, 604–608.

23. Vigorito, E., Perks, K.L., Abreu-Goodger, C. *et al.* (2007) microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. *Immunity* **27**, 847–859.
24. Xiao, C., Calado, D.P., Galler, G. *et al.* (2007) MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell* **131**, 146–159.
25. Johnnidis, J.B., Harris, M.H., Wheeler, R.T. *et al.* (2008) Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. *Nature* **451**, 1125–1129.
26. Vinuesa, C.G., Tangye, S.G., Moser, B. and Mackay, C.R. (2005) Follicular B helper T cells in antibody responses and autoimmunity. *Nature Reviews Immunology* **5**, 853–865.
27. Yu, D., Tan, A.H., Hu, X. *et al.* (2007) Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. *Nature* **450**, 299–303.
28. Xiao, C., Srinivasan, L., Calado, D.P. *et al.* (2008) Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes. *Nature Immunology* **9**, 405–414.
29. Vinuesa, C.G. and Goodnow, C.C. (2004) Illuminating autoimmune regulators through controlled variation of the mouse genome sequence. *Immunity* **20**, 669–679.
30. Vinuesa, C.G., Cook, M.C., Angelucci, C. *et al.* (2005) A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature* **435**, 452–458.
31. Ansar Ahmed, S., Penhale, W.J. and Talal, N. (1985) Sex hormones, immune responses, and autoimmune diseases. Mechanisms of sex hormone action. *American Journal of Pathology* **121**, 531–551.
32. Akkaraju, S., Ho, W.Y., Leong, D. *et al.* (1997) A range of CD4 T cell tolerance: partial inactivation to organ-specific antigen allows nondestructive thyroiditis or insulitis. *Immunity* **7**, 255–271.
33. Chtanova, T., Tangye, S.G., Newton, R. *et al.* (2004) T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells. *Journal of Immunology* **173**, 68–78.
34. Kim, C.H., Lim, H.W., Kim, J.R. *et al.* (2004) Unique gene expression program of human germinal center T helper cells. *Blood* **104**, 1952–1960.
35. King, C., Tangye, S.G. and Mackay, C.R. (2008) T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annual Review of Immunology* **26**, 741–766.
36. Radic, M.Z. and Weigert, M. (1994) Genetic and structural evidence for antigen selection of anti-DNA antibodies. *Annual Review of Immunology* **12**, 487–520.
37. Strasser, A. and Bouillet, P. (2003) The control of apoptosis in lymphocyte selection. *Immunological Reviews* **193**, 82–92.
38. Pugh-Bernard, A.E., Silverman, G.J., Cappione, A.J. *et al.* (2001) Regulation of inherently autoreactive VH4-34 B cells in the maintenance of human B cell tolerance. *Journal of Clinical Investigation* **108**, 1061–1070.
39. Anderson, M.S., Venanzi, E.S., Klein, L. *et al.* (2002) Projection of an immunological self shadow within the thymus by the aire protein. *Science* **298**, 1395–1401.
40. Liston, A., Lesage, S., Wilson, J. *et al.* (2003) Aire regulates negative selection of organ-specific T cells. *Nature Immunology* **4**, 350–354.
41. Sakaguchi, N., Takahashi, T., Hata, H. *et al.* (2003) Altered thymic T-cell selection due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. *Nature* **426**, 454–460.
42. Bachmaier, K., Krawczyk, C., Kozieradzki, I. *et al.* (2000) Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature* **403**, 211–216.
43. Naramura, M., Jang, I.K., Kole, H. *et al.* (2002) c-Cbl and Cbl-b regulate T cell responsiveness by promoting ligand-induced TCR down-modulation. *Nature Immunology* **3**, 1192–1199.
44. Walker, L.S. and Abbas, A.K. (2002) The enemy within: keeping self-reactive T cells at bay in the periphery. *Nature Reviews Immunology* **2**, 11–19.
45. Ueda, H., Howson, J.M., Esposito, L. *et al.* (2003) Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* **423**, 506–511.

46. Nagata, S. (1998) Human autoimmune lymphoproliferative syndrome, a defect in the apoptosis-inducing Fas receptor: a lesson from the mouse model. *Journal of Human Genetics* **43**, 2–8.
47. Ziegler, S.F. (2006) FOXP3: of mice and men. *Annual Review of Immunology* **24**, 209–226.
48. Malek, T.R. (2008) The biology of interleukin-2. *Annual Review of Immunology* **26**.
49. Bernard, D., Hansen, J.D., Du Pasquier, L. *et al.* (2007) Costimulatory receptors in jawed vertebrates: conserved CD28, odd CTLA4 and multiple BTLAs. *Developmental and Comparative Immunology* **31**, 255–271.
50. Nurieva, R., Thomas, S., Nguyen, T. *et al.* (2006) T-cell tolerance or function is determined by combinatorial costimulatory signals. *EMBO Journal* **25**, 2623–2633.
51. Dong, C., Juedes, A.E., Temann, U.A. *et al.* (2001) ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* **409**, 97–101.
52. Dong, C., Temann, U.A. and Flavell, R.A. (2001) Cutting edge: critical role of inducible costimulator in germinal center reactions. *Journal of Immunology* **166**, 3659–3662.
53. McAdam, A.J., Greenwald, R.J., Levin, M.A. *et al.* (2001) ICOS is critical for CD40-mediated antibody class switching. *Nature* **409**, 102–105.
54. Tafuri, A., Shahinian, A., Bladt, F. *et al.* (2001) ICOS is essential for effective T-helper-cell responses. *Nature* **409**, 105–109.
55. Mak, T.W., Shahinian, A., Yoshinaga, S.K. *et al.* (2003) Costimulation through the inducible costimulator ligand is essential for both T helper and B cell functions in T cell-dependent B cell responses. *Nature Immunology* **4**, 765–772.
56. Wong, S.C., Oh, E., Ng, C.H. and Lam, K.P. (2003) Impaired germinal center formation and recall T-cell-dependent immune responses in mice lacking the costimulatory ligand B7-H2. *Blood* **102**, 1381–1388.
57. Grimbacher, B., Hutloff, A., Schlesier, M. *et al.* (2003) Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. *Nature Immunology* **4**, 261–268.
58. Hutloff, A., Buchner, K., Reiter, K. *et al.* (2004) Involvement of inducible costimulator in the exaggerated memory B cell and plasma cell generation in systemic lupus erythematosus. *Arthritis and Rheumatism* **50**, 3211–3220.
59. Yang, J.H., Zhang, J., Cai, Q. *et al.* (2005) Expression and function of inducible costimulator, on peripheral blood T cells in patients with systemic lupus erythematosus *Rheumatology (Oxford)* **44**, 1245–1254.
60. Kawamoto, M., Harigai, M., Hara, M. *et al.* (2006) Expression and function of inducible co-stimulator in patients with systemic lupus erythematosus: possible involvement in excessive interferon-gamma and anti-double-stranded DNA antibody production. *Arthritis Research & Therapy* **8**, R62.
61. Lafferty, K.J., Andrus, L. and Prowse, S.J. (1980) Role of lymphokine and antigen in the control of specific T cell responses. *Immunological Reviews* **51**, 279–314.
62. Matzinger, P. (1994) Tolerance, danger, and the extended family. *Annual Review of Immunology* **12**, 991–1045.
63. Carreno, B.M. and Collins, M. (2002) The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. *Annual Review of Immunology* **20**, 29–53.
64. Greenwald R.J. Freeman G.J. Sharpe A.H. (2005) The B7 family revisited. *Annual Review of Immunology* **23**, 515–548.
65. Suh, W.K., Tafuri, A., Berg-Brown, N.N. *et al.* (2004) The inducible costimulator plays the major costimulatory role in humoral immune responses in the absence of CD28. *Journal of Immunology* **172**, 5917–5923.
66. Rasheed, A.U., Rahn, H.P., Sallusto, F. *et al.* (2006) Follicular B helper T cell activity is confined to CXCR5(hi)ICOS(hi) CD4 T cells and is independent of CD57 expression. *European Journal of Immunology* **36**, 1892–1903.

67. Carballo, E., Lai, W.S. and Blackshear, P.J. (1998) Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. *Science* **281**, 1001–1005.
68. Carballo, E., Lai, W.S. and Blackshear, P.J. (2000) Evidence that tristetraprolin is a physiological regulator of granulocyte-macrophage colony-stimulating factor messenger RNA deadenylation and stability. *Blood* **95**, 1891–1899.
69. Ogilvie, R.L., Abelson, M., Hau, H.H. *et al.* (2005) Tristetraprolin down-regulates IL-2 gene expression through AU-rich element-mediated mRNA decay. *Journal of Immunology* **174**, 953–961.
70. Li, W., Gao, B., Lee, S.M. *et al.* (2007) RLE-1, an E3 ubiquitin ligase, regulates *C. elegans* aging by catalyzing DAF-16 polyubiquitination. *Developmental Cell* **12**, 235–246.
71. Hudson, B.P., Martinez-Yamout, M.A., Dyson, H.J. and Wright, P.E. (2004) Recognition of the mRNA AU-rich element by the zinc finger domain of TIS11d. *Nature Structural & Molecular Biology* **11**, 257–264.
72. Laroia, G., Sarkar, B. and Schneider, R.J. (2002) Ubiquitin-dependent mechanism regulates rapid, turnover of AU-rich cytokine mRNAs. *Proceedings of the National Academy of Sciences USA* **99**, 1842–1846.
73. Paschoud, S., Dogar, A.M., Kuntz, C. *et al.* (2006) Destabilization of interleukin-6 mRNA requires a putative RNA stem-loop structure, an AU-rich element, and the RNA-binding protein AUF1. *Molecular and Cellular Biology* **26**, 8228–8241.
74. Anderson, P. and Kedersha, N. (2008) Stress granules: the Tao of RNA triage. *Trends in Biochemical Sciences* **33**, 141–150.
75. Scheu, S., Stetson, D.B., Reinhardt, R.L. *et al.* (2006) Activation of the integrated stress response during T helper cell differentiation. *Nature Immunology* **7**, 644–651.
76. Filipowicz, W. (2005) RNAi: the nuts and bolts of the RISC machine. *Cell* **122**, 17–20.
77. Kedersha, N., Stoecklin, G., Ayodele, M. *et al.* (2005) Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. *Journal of Cell Biology* **169**, 871–884.
78. Newbury, S.F., Muhlemann, O. and Stoecklin, G. (2006) Turnover in the Alps: an mRNA perspective. Workshops on mechanisms and regulation of mRNA turnover. *EMBO Reports* **7**, 143–148.

Abstract

Evidence gathered in the last few years has revealed how a class of small non-coding RNAs – microRNAs (miRNAs) – fine-tune gene expression post-transcriptionally, influencing the immunological repertoire, the size of mature lymphoid compartments and the threshold for lymphocyte selection and activation. More recently, the miRNA machinery has been shown to play an instrumental role in both the prevention and promotion of autoimmunity. Specifically, the Roquin pathway has emerged as a powerful negative regulator of systemic and organ-specific autoimmunity through miRNA-mediated control of T cell-expressed genes involved in T cell activation. Here we survey the evidence that miRNAs regulate immune responses relevant to the pathogenesis of autoimmunity and describe how Roquin acts in concert with T cell-expressed miRNAs to prevent lupus development.

Key words: microRNA; small non-coding RNA; autoimmunity; lupus; SLE; Roquin

16

Autoimmune response to post-translationally modified (citrullinated) proteins: prime suspect in the pathophysiology of rheumatoid arthritis

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16.1 Introduction

Rheumatoid arthritis (RA) affects 0.5–1% of the world's adult population with a 1:3 male to female ratio. It is characterized by chronic inflammation of peripheral articulations, frequently associated with systemic manifestations. In joints, synovial membrane abnormalities include hyperplasia of synoviocytes, capillaries and venules, and infiltration by immune cells jointly leading to formation of a pannus which erodes subjacent cartilage and bone, jeopardizing joint integrity and functional ability. The exact cause of RA is unknown, but joint inflammation is thought to be the result of an uncontrolled autoimmune response arising from a combination of environmental and genetic factors.

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16.2 RA is associated with B cell autoreactivity to citrullinated proteins

From the anti-perinuclear factor and anti-keratin antibodies ...

The presence of autoantibodies of diverse specificities in the serum of patients with RA constitutes a hallmark of the disease's autoimmune character. In particular, autoantibodies to citrullinated proteins (ACPA) are the RA-associated autoantibodies that have most focused the attention of both clinical and fundamental researchers over the last two decades. ACPA were initially described more than 40 years ago as the anti-perinuclear factor (APF), corresponding to autoantibodies specifically present in RA sera and labelling perinuclear granules in the most superficial keratinocytes of the human buccal epithelium by indirect immunofluorescence [1]. However, it was not until the mid-1990s that it was shown that the targeted antigen corresponded to a set of water-soluble variants of filaggrin, specifically expressed in the buccal epithelium [2]. Simultaneously, it was recognized that APF largely overlapped with the so-called anti-keratin antibodies (AKAs) [2], also RA-associated IgGs, labelling the cornified layer of the rat oesophagus epithelium by indirect immunofluorescence. Indeed, the initial description of AKAs had occurred 15 years later than that of APF [3], but their antigenic targets had been more quickly characterized, leading to the observation that they actually were not keratins but corresponded to water-soluble acidic–neutral isoforms of filaggrin present in the rat oesophagus epithelium and in the human epidermis [4,5].

In the epidermis, filaggrin is synthesized in the granular layer as a high-molecular-weight phosphorylated precursor, profilaggrin, stored in the cytoplasmic keratohyalin granules and corresponding to 10–12 tandemly repeated filaggrin units separated by linker peptides. During cornification, profilaggrin is dephosphorylated and cleaved by proteases, releasing filaggrin units. These filaggrin units, which are basic due to a high content in cationic amino-acyl residues, interact with anionic domains of keratin intermediate filaments, allowing their aggregation. Secondarily, they are processed into more acidic variants, inducing their dissociation from the keratin matrix and their complete proteolysis to generate free amino acids involved in the formation of the so-called natural moisturizing factor, a complex mixture of amino acid derivatives essential for the maintenance of epidermal hydration and photoprotection.

...to ACPA and powerful diagnostic assays

The acidification of filaggrin units occurs by conversion of the positively charged guanidino group of arginyl residues into an uncharged ureido group, thereby forming citrullyl residues (Figure 16.1). This post-translational transformation corresponds to a deimination, often called citrullination. It is catalysed by a Ca^{2+}-dependent enzyme,

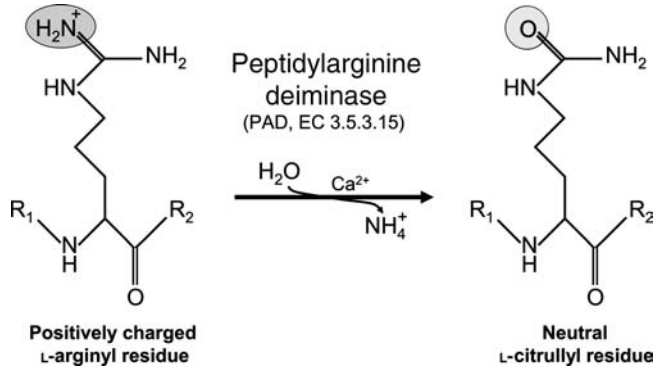

Figure 16.1 The deimination or citrullination reaction. It corresponds to the conversion of an arginyl residue of a peptide to form a citrullyl residue and ammonia. This transformation is catalysed by calcium-dependent enzymes designated peptidylarginine deiminase (PAD; protein-arginine deiminase, protein-L-arginine iminohydrolase, EC 3.5.3.15). R1 and R2 refer to amino-acyl residues involved in a peptide bond with the targeted arginyl residue.

designated peptidylarginine deiminase (PAD; protein-arginine deiminase, protein-L-arginine iminohydrolase, EC 3.5.3.15) of which five isotypes (PAD1, 2, 3, 4 and 6), encoded by five paralogous genes (*PADI*1–4 and *PADI*6) clustered on chromosome 1p35–36, have been described in humans [6,7]. As yet, knowledge on the expression patterns of the five PAD isotypes is not comprehensive, particularly for human tissues. PAD1 has been detected in the epidermis, hair follicles, arrector pili muscles and sweat glands [8,9]. PAD2 has been detected in brain astrocytes, sweat glands, arrector pili muscles, macrophages and epidermis [8,10–14]. PAD3 has been detected in the epidermis and in hair follicles [8,9,15,16]. PAD4 is present in haematopoietic cells [14,17–19] and is the only PAD isotype capable of nuclear translocation. PAD6 has been detected in total extracts of human ovaries [20], fitting with observations in the mouse where PAD6 expression occurs in the immature oocyte [21]. Because it decreases the net charge of protein targets, citrullination can lead to alterations in intra- and intermolecular interactions [22–24]. Even though its physiological role is not yet fully understood, it is clearly involved in terminal differentiation of epidermis and skin appendages [8,9,16,25] and in brain development [26,27]. It is essential for oocyte cytoskeletal sheet formation and preimplantation embryonic development [28,29], and it possibly plays a role in the regulation of gene expression by antagonizing methylation of histones through conversion of monomethylated arginine into citrulline [30–32]. Citrullination is also highly suspected of playing a pathophysiological role in RA, as will be discussed below. In addition, it is probably involved in the pathophysiology of multiple sclerosis and has been suggested to be implicated in Alzheimer's disease, primary open-angle glaucoma, psoriasis or obstructive nephropathy (for a review see [7]).

The observation that the (pro)filaggrin-related antigenic targets of AKA and APF in the various epithelial tissues exhibited a neutral/acidic pI suggested involvement

of a post-translational modification in the formation of the recognized epitopes, all the more so as recombinant filaggrin units expressed in *Escherichia coli* were not reactive with AKA-/APF-positive RA sera [33]. Both phosphorylation of serine residues or citrullination of arginyl residues were candidate post-translational modifications for playing that role, but citrullination proved to be critical. Indeed, it was demonstrated that autoantibodies reacting with human filaggrin-derived synthetic peptides containing citrullyl residues were specifically present in the serum of RA patients and that, when affinity-purified, such autoantibodies were positive in indirect immunofluorescence assays for APF and AKA detection and reactive to filaggrin extracted from human epidermis [34]. In addition, using an antibody specifically developed to react with all citrullinated proteins tested, hereafter referred to as 'anti-citrulline antibody' [35], it was shown that all the filaggrin-related targets of APF and AKA corresponded to citrullinated proteins and that *in vitro* citrullination of a recombinant human filaggrin by a PAD generated the epitopes recognized by AKA-/APF-positive RA sera on the protein [33]. Finally, in total, three independent studies have led to the identification of filaggrin-derived synthetic peptides specifically recognized by the autoantibodies only when their arginyl residue was substituted by a citrullyl residue [33,34,36]. In the three studies, however, not all citrullinated filaggrin-derived peptides proved to be equally immunoreactive with ACPA, showing that amino-acyl residues other than citrullyl residues also are involved in the formation of ACPA epitopes and therefore suggesting that ACPA are not the result of an autoreactivity directed to any citrullinated protein but rather of an immune response triggered by a single or a limited set of citrullinated antigen(s).

The tight association of RA with both AKA and APF was acknowledged early on [1,3,37–47]. The characterization of their antigen targets in epithelial tissues and the recognition of the importance of citrullination in epitope formation paved the way for the development of several assays for the detection of ACPA, first by immunoblotting and then by ELISA (see [48] for a review). The first commercially available test was based on the recognition of a citrullinated synthetic peptide derived from the 304–324-amino acid sequence of human filaggrin [34] and in which substitution of two seryl residues by cysteyl residues permitted cyclization by disulphide bonding (cyclic citrullinated peptide 1, CCP1) and improved the diagnostic performance [49]. Since then, other tests, all using citrullinated antigens, some of them of undisclosed nature, have been put on the market (see [50] for a recent comparative evaluation of the diagnostic performances of these assays). This development of modern assays replacing the not easily standardizable indirect immunofluorescence methods for the detection of ACPA greatly contributed to the acknowledgment of their diagnostic interest. Indeed, compared to indirect immunofluorescence methods, most of these new assays exhibited much higher diagnostic sensitivity with equivalent diagnostic specificity [48] (Figure 16.2, Clinical interest panel), leading to the recognition that between two-thirds and three-quarters of RA patients have ACPA in their serum at titres reaching values that are found in less than 2% of sera from patients with other non-RA rheumatic diseases.

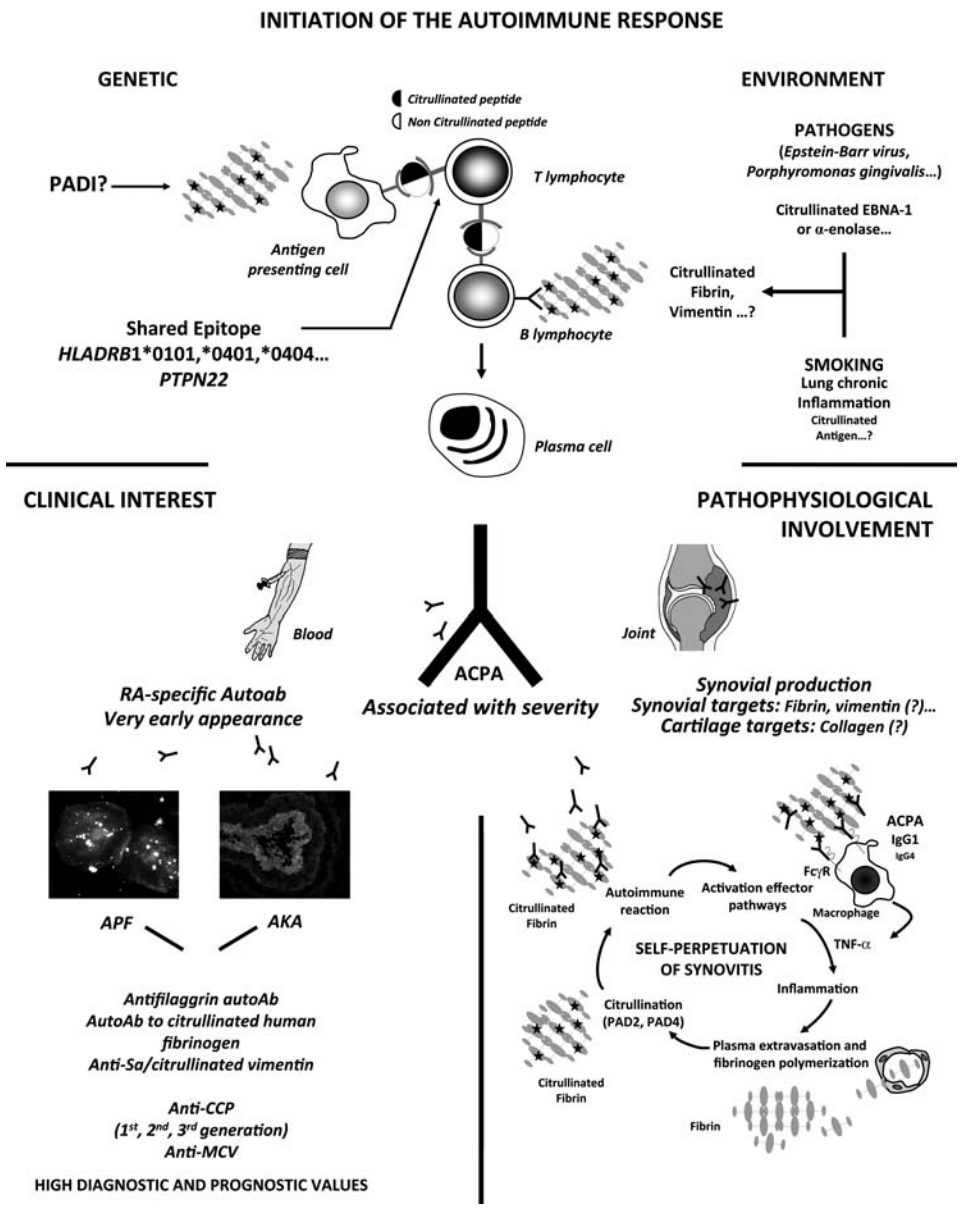

Figure 16.2 Possible events leading to the development of the autoimmune response to citrullinated proteins and the clinical and pathophysiological implications. ACPA production is summarized schematically in the upper panel. The lower left panel lists key features underlying the clinical interest of ACPA detection and the most notorious assays for their detection. The lower right panel proposes mechanisms by which ACPA could be involved in the self-perpetuation of RA synovitis. AutoAb, autoantibody; CCP, cyclic citrullinated peptide; EBNA-1, Epstein–Barr nuclear antigen 1; MCV, mutated citrullinated vimentin; PAD, peptidylarginine deiminase; *PADI*, gene encoding a peptidylarginine deiminase; *PTPN22*, gene encoding the protein tyrosine phosphatase non-receptor 22.

16.3 Both ACPA and citrullinated antigenic targets are present in the RA synovium

This very specific association of ACPA to most RA cases suggested that they may play a role in the pathophysiology of the disease, a hypothesis that then clearly needed further work to be substantiated. Because epithelial tissues are not usually targeted by rheumatoid inflammation, and since filaggrin, a protein marker of epithelial differentiation, was not expected to be expressed in the joints, it was likely that reactivity of citrullinated filaggrin with ACPA, though very useful for developing assays for their detection, was simply due to the fact that it shared epitopes with another autoantigen, putatively located into the synovial joint and corresponding to a genuine ACPA target *in vivo*. In 2000, this hypothesis was strengthened by the proof that the rheumatoid synovial tissue is a site for ACPA concentration since the proportion of ACPA among total IgGs is five to 10 times greater in the synovial membrane than in the serum [51]. Furthermore, it was demonstrated that ACPA are produced within the rheumatoid synovial membrane, as secretion of ACPA can be observed in the supernatant of RA synovial tissue fragments maintained *in vitro* several weeks after their excision from the joint [51], an observation that was confirmed a few years later by detecting ACPA in the serum of SCID mice engrafted with human synovial tissue fragments from ACPA-positive RA patients [52]. It was also formally demonstrated that (pro)filaggrin is not expressed in articular tissues [53]. Moreover, an extensive immunochemical analysis of rheumatoid synovial tissue proteins in search for citrullinated antigens that would be recognized by ACPA revealed that rheumatoid synovial membranes contain several citrullinated proteins, among which only two proteins, of 64–68 and 55–61 kDa, respectively, were specifically targeted by a significant proportion of ACPA-positive sera from RA patients and by affinity-purified ACPA [53]. In that same study, N-terminal sequencing and the use of fibrin-specific antibodies identified the two 64–68 and 55–61 kDa proteins to the α- and β-chains of fibrin, respectively. Moreover, ACPA-positive RA sera and purified ACPA were found to become highly reactive to the Aα- and Bβ-chains of human fibrinogen only after it was citrullinated *in vitro* by a PAD. Finally, the fact that both the autoantibodies to the citrullinated Aα- and Bβ-chains of fibrinogen and the autoantibodies to the citrullinated 64–68 and 55–61 kDa synovial proteins largely overlapped with ACPA was established by showing that autoantibodies affinity-purified from a pool of RA sera on a citrullinated fibrinogen column were reactive towards all the so-far-identified epithelial and synovial targets of ACPA [53]. These results established citrullinated forms of fibrin, histologically observable in the rheumatoid synovial membranes as both citrulline- and fibrin-positive interstitial amorphous deposits [54], as a major synovial target of ACPA. Moreover, for the first time, it was demonstrated that highly prevalent RA-specific autoantibodies and their autoantigenic targets were simultaneously present in the synovial tissue, most probably leading to an autoimmune reaction located in the organ predominantly affected by the disease.

16.4 Autoreactivity to citrullinated proteins probably plays a role in RA synovitis

These important findings constituted a major argument for considering the autoreactivity to citrullinated proteins involved in the pathophysiology of RA. Numerous other independent studies have helped to end the view that such autoreactivity is just a side effect of this disease. An indirect argument is the clinical efficacy of treating RA with rituximab, a chimaeric anti-CD20 monoclonal antibody that depletes naïve and memory B cell subsets. Although it remains unclear whether the sustained clinical remission it induces is due to a decrease in antigen-presenting and T cell-activating functions of B cells, or to a reduction in the secretion of autoantibodies or to both phenomena, such clinical improvement unquestionably contributed to demonstrating the major role of B cells in the immunopathogenesis of RA [55]. Another hint that autoantibodies found in RA have arthritogenic potential came with the observation that IgGs fractions of sera or plasmas from RA patients, but not from healthy individuals, were able to induce inflammation and histological lesions in the ankles of mice deficient in the inhibitory FcγRIIB IgGs receptor, when injected into the peritoneum [56]. More directly concerning ACPA, and suggesting that they are not neutral to the disease course, is the notion that the presence of ACPA in the serum is not a consequence of chronic synovitis since it occurs early in the disease course [57–69] and, further, frequently predates for several years the appearance of clinical signs of RA, a fact acknowledged in the early 1990s when the antibodies were still called AKA [70], and confirmed later with the first commercially available ACPA assays [71–73]. In addition a positive association of their presence and/or titre with the intensity of radiologically observed joint damage has been reported in numerous not only cross-sectional [74–83], but also longitudinal [64,68,84–97] studies, unravelling the high prognostic value of these powerful diagnostic markers.

Furthermore, indications of the deleterious effect of ACPA came from studies in the widely used animal model of RA, collagen-induced arthritis (CIA), induced generally in mice or rats by immunization with an autologous or a heterologous form of the cartilage matrix component collagen type II (CII) in adjuvant. Indeed, in the LEW.1AV1 rat strain it was shown that in comparison to autologous non-citrullinated CII, autologous citrullinated CII induced arthritis with an earlier-onset and a higher incidence and tended to have increased severity [98]. In addition, it was observed that DBA/1J mice with CIA develop IgGs antibodies specific for the citrullinated forms of proteins and peptides (among which is a citrullinated form of the β-chain of human fibrinogen), appearing early after immunization with bovine CII, even before joint swelling [99]. These data contradict previous reports on the absence of citrulline-specific autoantibodies in streptococcal cell wall-induced arthritis and CIA, investigated by an anti-CCP ELISA and using *in vitro*-citrullinated fibrinogen as immunosorbent [100,101]. Moreover, the biochemical nature of the autoantigen(s) these antibodies recognize in arthritic mice is still elusive and cross-reactivity with mouse citrullinated fibrinogen has not been explored. Nevertheless, their arthritogenic potential was

indicated by the fact that mouse monoclonal antibodies reactive with citrullinated human fibrinogen derived from mice with CIA enhance arthritis when administered simultaneously to a submaximal dose of a pool of anti-CII antibodies [99]. Moreover, mice tolerized with a citrulline-containing peptide do not produce the citrulline-specific autoantibodies when immunized with CII and the CIA they develop exhibits reduced incidence and severity [99]. Finally, very recently, in mice transgenic for the RA-associated major histocompatibility complex (MHC) class II molecule DRB1*0401 (DR4-IE tg mice), it was shown that induction of an autoimmune response to human citrullinated fibrinogen by direct immunization with this antigen could provoke an arthritis characterized by synovial hyperplasia with some lymphocyte infiltration and pannus formation at bone and cartilage interfaces, in 35% of the animals. On the other hand, arthritis was never observed in DR4-IE tg mice immunized with non-citrullinated human fibrinogen or in wild-type C57BL/6 mice immunized with citrullinated fibrinogen [102]. In addition, in this model it was found that human citrullinated fibrinogen elicited citrulline specific T cells that induced arthritis when transferred into the peritoneum of naïve DR4-IE tg mice that received an intra-articular injection of human citrullinated fibrinogen. Should these data be confirmed, their importance resides not only in the fact that they identify immune responses to a human synovial target of ACPA as arthritogenic but also in the recognition that this arthritogenic potential requires the presence of MHC class II molecules that in humans have been identified as RA-associated or, more accurately, as will later be re-evoked, as factors predisposing to the ACPA response.

16.5 The way ACPA could promote joint inflammation

As mentioned above, it is likely that the participation of ACPA in the RA pathophysiology involves their interaction with an antigen target present in the articular tissues. The isotype profile of ACPA, mainly corresponding to IgG1 frequently associated with IgG4 [103,104], is perfectly compatible with a capacity to form immune complexes able to activate complement- and Fcγ receptor (FcγR)-dependent pathways, potentially leading to production of inflammatory cytokines. Fitting with this assumption is the fact that a recently developed human *in vitro* model demonstrated the pro-inflammatory potential of the interaction of ACPA-containing immune complexes via engagement of FcγR at the surface of rheumatoid synovial tissue macrophages [105]. The study showed that macrophages derived from monocytes of healthy blood donors secrete tumour necrosis factor (TNF)-α when stimulated by *in vitro*-reconstituted ACPA-containing immune complexes, generated by capturing ACPA on immobilized citrullinated fibrinogen. Moreover, the FcγRIIa receptor was found to be the major activating FcγR involved in macrophage activation. This result is particularly interesting in view of the fact that synovial macrophages contribute considerably to inflammation and joint destruction in RA, precisely because they constitute the major articular source of TNF-α [106], a key cytokine in these two aspects of the disease [107]. Moreover, these data fit with

numerous reports that have demonstrated the arthritic potential of FcγR-mediated cell responses, notably through study of induction of experimental arthritis in mice deficient in different types of FcγR [108].

16.6 Joint-expressed citrullinated autoantigen targets possibly involved in a pro-inflammatory effect of ACPA

Vimentin

Since the discovery that rheumatoid synovial joints contain citrullinated proteins, various studies have aimed to identify these proteins and the precise targets of ACPA in the RA joints that can engage in a synovitis-promoting autoimmune reaction. After citrullinated fibrin was proposed to play that role [53], citrullinated vimentin was the other candidate citrullinated autoantigen to be put forward first. In 2004 it was demonstrated that anti-Sa autoantibodies, described 10 years earlier as tightly RA-associated autoantibodies [109], were actually reactive to a citrullinated variant of vimentin [110]. It then became tempting to speculate that an autoimmune reaction between ACPA and citrullinated vimentin, occurring within the synovial tissue, played a role in RA synovitis [111–113], all the more so as citrullination of vimentin had been shown to occur in macrophages upon induction of apoptosis by calcium influx [14,114]. However, until very recently peer-reviewed data on the mere presence of citrullinated vimentin in RA joints had not been published. In 2008, the presence of mutated and citrullinated forms of vimentin in a pool of synovial fluid samples from RA patients was shown by mass-spectrometric analysis of vimentin-enriched fractions and it was proposed, but not compellingly demonstrated, that oxidative stress could be the underlying trigger for the generation of these vimentin variants [115]. Subsequently an ELISA was developed using mutated citrullinated vimentin as immunosorbent and, in comparison to the citrullinated form of wild-type vimentin, was found to exhibit enhanced antibody reactivity in a small series of RA sera. However, it was not examined whether the rise in sensitivity obtained when using mutated citrullinated vimentin is achieved without a concomitant loss of specificity. Nevertheless, based on these results, an ELISA using mutated citrullinated vimentin as immunosorbent has been developed [115] and is now commercially available. Remarkably, as of today, among various studies comparing the diagnostic performance of this ELISA with that of a second-generation ACPA assay using cyclic citrullinated peptides (CCP2 test), reports of a superior diagnostic performance of the mutated citrullinated vimentin assay correspond to evaluation of the assays using sera from healthy individuals as controls [115,116]. On the other hand, when controls correspond to sera from patients with a non-RA disease, a lower diagnostic sensitivity at identical specificity thresholds is observable [117,118]. Finally, using a proteomic approach a very recent study has shown the presence of a processed (probably caspase-3-cleaved) form of vimentin among the Tris-extractible proteins of the synovial tissue from three out of 11 patients with RA. Moreover, the presented data suggested that this vimentin variant was

citrullinated and recognized by IgGs specifically present in the serum of RA patients, indicating that a disease-specific autoimmune reaction involving this autoantigen may form in the synovium of at least some RA patients [119].

Collagen

The capacity of prominent joint protein markers to be citrullinated *in vitro* and thereby to become antigens recognized by IgGs from RA sera has also been addressed. In particular, the reactivities to citrullinated forms of collagen type I (CI), an important component of the bone matrix, and of the cartilage component CII have been investigated by several groups [120–123]. In 2005, by immunoscreening *in vitro*-citrullinated proteins encoded by an RA synoviocyte cDNA library with the 'anti-citrulline antibody', the α1 and α2 chains of CI were shown to be PAD substrates. Moreover, by ELISA screening with sera from healthy individuals, and from RA and non-RA patients, reactivity to *in vitro*-citrullinated CI was found to be specifically higher in RA sera and to correlate with the levels of ACPA measured by a CCP ELISA [120]. Remarkably, in the same study, an ELISA screening of *in vitro*-citrullinated CII detected no significant difference between the reactivity of RA sera and that of sera from either normal controls or non-RA patients. On the other hand, also in 2005, in a cohort of 286 early RA patients, IgGs antibodies reacting with a synthetic citrullinated triple-helical peptide containing a previously identified non-citrullinated epitope encompassing residues 359–369 of CII, were detected with a prevalence of 40.4% [121]. However, in some sera this reactivity could be inhibited using a non-citrullinated form of the peptide and the citrulline-dependence of the reactivity to the peptide was not systematically investigated in all serum samples. Nonetheless, in 2006, another study reported detection of autoantibodies to an *in vitro*-citrullinated form of full-length CII in 78.5% of 130 serum samples from RA patients whereas autoantibodies to non-citrullinated CII were present in only 14.6% of serum samples. In addition, reactivity to citrullinated CII was found in only three of 170 sera from non-RA patients [123]. Finally, the presence in RA patients of circulating autoantibodies recognizing citrullinated peptides derived from the sequence of the C-terminal extremities of the α1 and α2 chains of CI and of the α1 chain of CII has also been described, but the actual prevalence at sensitivity threshold allowing high diagnostic specificities (>98%) to be reached has not been clearly ascertained [122,124–127]. Obviously, these data point to other possible antigenic targets of ACPA in the RA joint of potentially high pathophysiological significance, invigorating the relevance of studying autoimmune responses to collagen in CIA as an appropriate model of pathogenic autoimmune mechanisms in human RA. However, it seems too early to speculate on the matter before the actual presence of more than minute amounts of the citrullinated forms of these antigens in the joints of RA patients has been convincingly demonstrated. In that respect, it seems important to mention that *in vitro* citrullination of CII seems to require exceptionally high amounts of PAD since around 100 units of commercially available rabbit PAD2 per milligram of human CII was used in the studies mentioned above when at least around 10 times less enzyme is usually employed for

other substrates, raising the question as to whether citrullination of cartilage collagen can actually occur *in vivo*.

Fibrin

To try and identify citrullinated autoantigens in the joint of patients with RA, proteomic approaches have also been undertaken [119,128,129]. Extracts of the synovial membrane or of exosomes present in the synovial fluid were analysed by two-dimensional electrophoresis and immunoblotting using the anti-citrulline antibody and/or sera from RA patients, followed by identification of immunoreactive spots using mass spectrometry analyses. As a result, a few previously uncharacterized citrullinated proteins have been proposed to serve as targets for ACPA [128,129]. However, so far the prevalence of autoreactivity to these proteins in RA sera either remains to be convincingly evaluated or is rather low, raising the possibility that these proteins are only targeted by a subset of ACPA because they serendipitously harbour some of the epitopes recognized by ACPA. On the other hand, it is interesting to note that citrullinated fibrin has recurrently been pinpointed as a candidate target antigen by proteomic approaches [119,128,129]. Moreover, in three independent studies, the diagnostic sensitivity for RA of assays for ACPA detection corresponding to ELISAs using *in vitro*-citrullinated fibrinogen as immunosorbent have been found to be similar to that of the most sensitive commercially available ACPA assay (then the CCP2 ELISA) when both tests are compared at equally high diagnostic specificities (>95%) [64,67,130]. In addition, sequential epitopes recognized on fibrin by ACPA have been identified by ELISA screening of 71 citrullinated 15-mer peptides derived from all the sites of the α- and β-chains of fibrin harbouring arginyl residues [131]. This study provided a very strong confirmation of the importance of the amino-acyl environment of citrullyl residues in epitope formation as only 18 of the 71 citrullinated fibrin-derived peptides were specifically recognized by ACPA-positive RA sera. Additionally, it was found that two out of the 18 peptides, located in the central globular domain of fibrin, bear highly dominant ACPA epitopes as they are not only recognized by a high proportion of RA sera but are also able conjointly to almost abolish the reactivity to *in vitro*-citrullinated fibrinogen of a highly polyclonal mixture of ACPA derived from the serum of 38 different RA patients [105,131]. Finally, it has long been known that, in the inflamed RA synovium, there is ongoing extravascular fibrin formation that correlates with the degree of inflammation [132] and, although the fact that fibrin could be citrullinated was only acknowledged upon discovery that it could constitute a target for ACPA in the RA synovial membrane, it was quickly observed that it was also a general phenomenon in actively inflamed RA synovial tissues [54].

It therefore appears that citrullinated fibrin up to now constitutes the sole autoantigen that clearly reunites all the necessary characteristics that should be possessed by any antigen likely to embark in a disease impacting autoimmune reaction with ACPA into the synovial joints of RA patients: its frequent presence in the RA synovial membrane has been demonstrated conclusively and the autoantibodies recognizing it

probably encompass the whole ACPA family, meaning that autoantibodies able to interact with this autoantigen are present in virtually all ACPA-positive patients. Furthermore, a peculiarity of fibrin makes it a good candidate for playing an important role in the self-perpetuating character of RA synovitis. Indeed, as mentioned previously, fibrin formation in the synovial tissue is an inflammation-dependent phenomenon resulting from the hyperpermeability of the inflamed synovial microvasculature, leading to extravasation of plasma fibrinogen rapidly clotted to fibrin, notably due to inflammation-induced expression of tissue factor that mediates activation of the extrinsic coagulation pathway [133]. As the disease-specific autoimmune reaction between ACPA and citrullinated fibrin activates effector mechanisms, the ensuing pro-inflammatory effects therefore promote formation of new fibrin deposits into the synovial membrane. These deposits can then be citrullinated by PADs whose expression in the synovial membrane also seems inflammation-induced, as will further be recounted. As a result, fibrin deposits become new targets for ACPA and that fuels the synovitis-perpetuating autoimmune reaction (Figure 16.2, Pathophysiological involvement panel). Of note, expression of PAD in the synovial membrane of RA patients has already been established [19,20,134–136]. Notably, in a comprehensive study systematically examining the five PAD isotypes, it was found that PAD2 and PAD4 but not PAD1, PAD3 and PAD6 were present, and that both enzymes were demonstrable within or in the vicinity of citrullinated fibrin deposits, suggesting that both isotypes are involved in their citrullination [20].

Fibronectin

Finally, it should also be mentioned that following an immunohistological study, the presence of citrullinated fibronectin has been described in the synovial tissue of RA patients [137], with the associated suggestion that fibronectin citrullination is associated to RA on the basis that extracellular aggregates of fibronectin were only detectable in the synovium of RA patients and not in that of patients with osteoarthritis. However, the absence of fibrin and fibronectin in control tissue samples was probably due to the fact that they were probably not inflamed since they were not selected on that criterion. Analysis of inflamed non-RA synovial tissue samples may have led to a different conclusion. Nevertheless, should the results be confirmed by immunochemical analyses, in RA synovial membranes the reported co-location of citrullinated fibronectin with citrullinated fibrin deposits suggests that citrullination of these two proteins, known to be able to associate via covalent factor XIII-catalysed ε-γ-glutamyl lysine bonds, could occur concomitantly in the inflamed synovium. It would therefore be interesting to formally test whether citrullinated fibronectin also constitutes an antigen target for ACPA, hence suggesting that it could also serve as an abundant locally expressed target for the autoantibodies and participate in the harmful autoimmune reaction involving ACPA.

16.7 Initial triggering of the autoimmune response to citrullinated proteins

The so-far-mentioned (possible) joint targets of ACPA constitute candidates for participating in autoimmune reactions and, possibly, for maintaining ACPA production, as the presence of ACPA of the IgM class in established IgGs ACPA-positive RA [138] suggests that recruitment of new B cells into the ACPA response is sustained along the disease. The possible mechanisms involved both in the initiation of this autoimmune response and in the fact that this specifically occurs in RA patients will now be discussed (Figure 16.2, panel on the initiation of the autoimmune response).

Citrullinated proteins as neoantigens?

The possibility that citrullination of autologous proteins could transform some of them into immunogenic neoantigens, because of the absence of previous education of the immune system able to induce immunological tolerance, has been tested in animals. In LEW.1AV1 rats, citrullination of autologous serum albumin made it able to elicit an IgGs antibody response upon inoculation with incomplete Freund's adjuvant whereas the native form was not immunogenic [98]. The immunogenic potentials of complete Freund's adjuvant-emulsified autologous citrullinated and non-citrullinated fibrinogen have also been comparatively evaluated in BALB/c mice [139] and Lewis and Brown-Norway rats [140]. Whereas non-citrullinated fibrinogen induced no antibody response, the citrullinated form triggered the development of an IgGs autoimmune response essentially directed to citrullinated determinants. It should however be noted that even though citrullinated fibrinogen readily induced a primary autoimmune response in Lewis rats, a secondary immune response could not be elicited, probably due to the existence of mechanisms able to exert strong negative controls on the developing immune response. The concomitant development of a response to non-citrullinated fibrinogen in rats inoculated with citrullinated fibrinogen could be responsible for the establishment of such mechanisms. Indeed, it was previously noted that among several strains of mice immunized with native human fibrinogen, the strains where the elicited immune response was the most cross-reactive with autologous fibrinogen coincided with the strains where no strong secondary response could be obtained after a second inoculation of the human antigen [141]. Be it as it may, the mouse and rat studies both agreed in the finding that the autoimmune response to citrullinated autologous fibrinogen could not spontaneously induce joint inflammation. This could be in relation to the establishment of the above-mentioned strong immunoregulatory mechanisms, which are probably not at work in DR4-IE tg mice that develop arthritis upon immunization with citrullinated human fibrinogen [102], and/or to the absence of an antigenic target in the synovial tissue of the immunized animals [140].

In line with the above-mentioned experimental approaches and because this could explain why ACPA are so tightly associated to RA, whether the presence of citrullinated fibrin in the synovial tissue is also specific for RA was investigated. In studies answering this question indirectly it was demonstrated that the presence of citrullinated proteins, including citrullinated fibrin, occurred in the synovial tissue of mice after induction of arthritis by intra-articular injection of streptococcal cell wall or by immunization with CII, while the serum of the animals remained devoid of ACPA [100,101]. In addition, an investigation of the presence of citrullinated proteins in the synovium of patients with RA or with other inflammatory or non-inflammatory disorders led to the observation, in the synovial tissue of both RA and control patients, of citrullinated proteins in extracellular areas considered as corresponding to fibrin deposits in the absence of any biochemical or immunological evidence [142]. A conclusive answer to the question was finally obtained by comparing synovial tissue samples from RA patients with those from non-RA inflammatory and non-inflammatory rheumatic diseases after selecting the samples for the presence of a synovitis, since it was expected that fibrin, citrullinated or not, would only be present in inflamed tissues [54]. Using antibodies to fibrin, ACPA purified from RA sera and the so-called anti-citrulline antibody, the immunoblotting and histological analyses conducted showed the presence of a variable quantity of citrullinated fibrin in all samples. Therefore, fibrin citrullination in the synovial tissue is a general phenomenon associated with any type of synovitis and not necessarily accompanied by induction of an autoimmune response with production of ACPA. Not surprisingly, the results of the three above-cited studies are concordant with results obtained when the presence of the enzymes responsible for citrullination was examined in the joint of animals with experimental arthritides or in that of RA patients. While both PAD2 and PAD4 enzymes could not be detected in the synovial tissue of naïve mice, PAD4 (and not PAD2) was evidenced in the inflamed synovial tissue of mice with CIA or with streptococcal cell wall-induced arthritis [100]. In human synovial tissues, systematic analysis of the expression of the five PAD isotypes found that PAD2 and PAD4 were present in samples not only from RA patients but also from patients with other arthritides and that PAD2 was also expressed in samples from patients with osteoarthritis [20]. Furthermore, the expression levels of PAD2 and PAD4 were both correlated with the intensity of inflammation, particularly the level of cell infiltration [20]. Finally, also fitting with all these observations, reports have been made of the presence of citrullinated proteins of still unknown identity in several other inflamed non-synovial tissues, such as rheumatoid nodules, the muscle of patients with polymyositis, the tonsil of patients with chronic tonsillitis or the lung of individuals with RA-associated or idiopathic interstitial pneumonia [143,144]. Therefore, in addition to its physiological occurrence as part of the differentiation programme in defined organs such as the skin and the central nervous system, citrullination also appears as able to affect probably any type of tissue subjected to inflammation, due to the induction of the expression and/or of the activity of PAD(s) in resident or infiltrating inflammatory cells. Consequently, it now seems highly reasonable to exclude the possibility that inflammation-induced expression, specifically in RA patients, of some citrullinated antigen target constitutes the direct explanation for their

specific autoimmunization against citrullinated proteins, all the more so as ACPA appear several years before onset of overt inflammation and development of the ACPA response in initially ACPA-negative patients is rarely seen after the disease is clinically manifest. A little doubt remains when considering a synovial membrane intracellular citrullinated antigen defined by a particular commercially available antibody to citrulline raised in rabbits by immunization with L-citrulline coupled to keyhole limpet haemocyanin. Very interestingly, it has repeatedly been demonstrated to be present only in the synovial tissue of RA patients and not in that of patients with other inflammatory or non-inflammatory rheumatologic disorders, and it was accordingly proposed to be a candidate for driving the RA-specific ACPA response [136,145,146]. Unfortunately, however, its biochemical nature remains as yet elusive, notably because the antibody to citrulline that defines it cannot be reliably used in immunochemical techniques. Such techniques would however permit conclusive demonstration of the recognition of this antigen by ACPA, an essential prerequisite to reinforce and further explore the hypothesis that it could play a role in their specific induction in RA patients.

A role for immune-response genes?

Given the very high prevalence of ACPA in RA, their high specificity for the disease, and their probable pathophysiological implications, many of the as yet incompletely defined and probably multiple combinations of environmental and genetic factors that predispose to RA may actually do so because they positively influence the development of the autoimmune response to citrullinated proteins. Regarding genetic influences, progress has recently been made concerning the impact of the gene encoding the MHC class II HLA-DRB1 chain. Thirty years ago, a strong association between RA and MHC class II genes was unravelled [147]. This association was subsequently largely confirmed and it was recognized that *HLA-DRB1* was the major susceptibility locus within the histocompatibility locus antigen (HLA) class II region [148], and that susceptibility alleles of this presenting molecule contained a conserved five-amino acid motif, called the shared epitope, that was proposed to be the distinctive characteristic conferring a dose-dependent predisposition to the disease [149]. Less than 10 years ago, an association between shared epitope-bearing alleles and the presence of ACPA in RA patients sera was reported in several independent studies [59,85,150–152]. Not long after, it appeared that the association of RA with *HLA-DRB1* alleles actually stood exclusively for the ACPA-positive subset, and not for the ACPA-negative subset and that shared epitope alleles constitute a risk factor rather for production of ACPA than for RA development [153–155]. One possible direct explanation for this is that shared epitope-bearing HLA-DR molecules have a particular capacity to present antigenic peptides derived from citrullinated proteins to T cells providing help to ACPA-producing B cells. In line with this hypothesis, it was reported that the substitution of an arginyl residue of a vimentin-derived peptide by a citrullyl residue induced a 10–100-fold increase in the affinity of the interaction with the shared epitope-positive *0101, *0401 and *0404 HLA-DR molecules while this did not occur with the shared epitope-

negative *0802, *1101 and *1302 variants [156]. However, *in vitro* evaluation of the interaction of purified HLA-DR *0401, *0404 and *0101 molecules with peptides encompassing the whole α-chain and the whole β-chain of fibrin and with all the citrullinated variants of these peptides revealed that every tested allele was capable of binding an important number of peptides and that citrullination did not systematically positively influence fibrin peptide binding to these HLA-DR molecules [152], suggesting that citrullination, even though pivotal in the formation of the epitopes recognized by ACPA, may not be an essential prerequisite for the recognition by T cells. Moreover, recognition of citrullinated peptides by T cells could be a common phenomenon associated with immunization, since it has recently been observed that immunization of mice with non-citrullinated hen egg-white lysozyme induced the activation of T cells specific for citrullinated epitopes and that, *in vitro*, mouse dendritic cells and peritoneal macrophages presented citrullinated peptides of hen egg-white lysozyme even when cultured in the presence of the native form of the antigen [157].

HLA-DRB1 is not the sole RA-associated immune response gene for which data have accumulated indicating that association to the disease is restricted to the ACPA-positive subset. For instance, the C1858T single nucleotide polymorphism in the *PTPN*22 gene encoding the haematopoietic-specific protein tyrosine phosphatase non-receptor 22 (also known as LYP) confers increased susceptibility to RA, among other autoimmune diseases [158]. Concerning RA, several studies have now reported that this polymorphism is actually specifically associated with the development of ACPA-positive RA [159–162].

*An influence of genes encoding PADs (*PADI *genes)?*

Given that PADs are involved in the generation of the antigenic targets of ACPA in the synovial tissue and since systematic genome analyses (linkage studies) have revealed a possible RA genetic factor in the region of chromosome 1 encompassing the *PADI* locus, *PADI* genes also constitute candidate genes for the development of ACPA-positive RA. In 2003, single nucleotide polymorphisms of the genomic region encompassing the *PADI*1–4 genes were explored in a case-control study within the Japanese population, leading to the discovery of an association between RA and a *PADI*4 haplotype [134]. The genetic association was confirmed in a replicate study from Japan [163], in a Korean population [164] and in Caucasian populations from North America [165] and Germany [166], but was not reproduced in Caucasian populations from the UK, France or Spain [167–170]. In the initial study, *in vitro* results suggested that the presence of the *PADI4* haplotype could lead to a more stable mRNA and the authors proposed that PAD4 expression was increased and that this could lead to enhanced levels of immunogenic citrullinated proteins [134]. However, the impact of the putative RA-associated *PADI*4 haplotype on PAD4 expression levels remains to be explored. Furthermore, in two studies where an association with RA was observed, no difference in the frequencies of susceptible haplotypes were shown between ACPA-positive and ACPA-negative RA patients [166,171], whereas one study reported that,

among ACPA-positive patients with early RA, ACPA levels were significantly higher in patients carrying the RA risk haplotype than in patients who did not carry it [172]. Therefore, the question of whether variants of the *PADI4* gene are associated with RA (or with ACPA-positive RA) is still open and the issue as to whether and how the *PADI*4 gene could constitute a genetic influence in triggering the autoimmune response to citrullinated proteins is not solved. Association of RA or of ACPA-positive RA with variants not only of the gene encoding PAD2, an isotype also expressed in the rheumatoid synovial membrane, but also of the *PADI*1, 3 and 6 genes, as the enzymes they encode may be at work in the citrullination of an initiating autoantigen in a different tissue, deserves further testing.

Environmental triggers?

The analysis of RA occurrence in both monozygotic and dizygotic twin pairs has shown that environmental factors also play a part in RA aetiology. Obviously, the possibility that they exert their influence by contributing to the development of the autoimmune response to citrullinated proteins should not be overlooked. In this respect some recently uncovered tracks deserve further attention. In particular, cigarette smoking was not only repeatedly found to constitute a risk factor for RA [173] but also to constitute an environmental factor strongly interacting with the HLA-DR shared epitope for the development of ACPA-positive RA [155]. Indeed, in RA patients from Sweden, previous smoking was dose-dependently associated with the occurrence of ACPA. Moreover, whereas smoking conferred a small increased risk for the development of ACPA-positive RA in shared epitope-negative individuals, smoking in the presence of one or two shared epitope alleles dramatically increased that risk [155]. This observation of a major gene–environment interaction between the *HLA-DRB1* gene and smoking in the production of ACPA has been replicated in Dutch and Danish populations [174,175] but not observed in three out of four studied American cohorts [176,177]. A model of RA aetiology was proposed, in which heavy smoking stimulates cell infiltration into the lungs, and subsequent activation and dying of these cells by apoptosis prompts PAD expression and activation, leading to citrullination of proteins. Presentation of citrullinated peptides from these proteins by shared epitope-bearing HLA-DR molecules then activates T cells that in turn provide help to ACPA-producing B cells [178]. To support this model, investigations have been performed on the presence of citrullinated proteins and of PAD2 and 4 in the lung of healthy smokers using immunohistological approaches [179]. This led to observation of an increased expression of citrullinated proteins in cells from bronchoalveolar lavage and of PAD2 in bronchial mucosal biopsies in comparison with samples from non-smoking healthy individuals. Should these results be confirmed and extended by immunochemical and biochemical approaches they could open the way to identification of the putative cigarette-induced antigen trigger for ACPA.

Besides lifestyle risks such as cigarette consumption, the hypothesis that infection is the initiator of the inflammatory process in RA is very longstanding and mainly

supported by indirect evidence [173]. Here we will concentrate on two microbes that could play a role in triggering the ACPA response. Both infectious agents might act as initiators because they express immunogenic antigens that can become citrullinated in the infected tissue or cell, thereby acquiring epitopes among which some exhibit sequential or structural homology with citrullinated self-antigens that, due to cross-reactivity of the immune reaction, then serve as targets for maintenance of both the synovitis and the immune response.

First, the Epstein–Barr virus (EBV) has been implicated as a potential risk factor for RA for over 25 years based on various pieces of supporting evidence (for a review see [180]). Recently, reactivity to a citrulline-substituted peptide derived from the N-terminal 35–58 amino-acyl residues of the virus-encoded Epstein–Barr nuclear antigen 1 (EBNA-1) has been evaluated in RA sera as well as in control sera including connective tissue diseases, chronic arthritides and healthy donors with the result that antibodies binding to the peptide were detected in 45–50% of RA sera and in less than 5% of the control sera [181,182]. In addition, the levels of antibodies targeting this peptide correlated with anti-CCP levels, and antibodies affinity-purified on the citrullinated viral peptide reacted with *in vitro*-citrullinated forms of fibrinogen [182]. Moreover, from the lysate of a calcium ionophore-stimulated EBV-transformed lymphoblastoid cell line, the purified antibodies could immunoprecipitate a 80 kDa protein that was identified as a citrullinated form of EBNA-1, based on its reactivity with a monoclonal anti-EBNA-1 antibody and with the so-called anti-citrulline antibody [182]. These interesting data suggest that citrullination of EBV proteins may occur in EBV-infected cells undergoing apoptosis and that, at least in a subset of RA patients, EBV infection may play a role in the induction of ACPA.

Second, by immunoblotting *in vitro*-citrullinated lysates of HL-60 cells after monocytic or granulocytic differentiation, a potential target of RA sera was detected that was subsequently identified to a citrullinated form of the glycolitic enzyme α-enolase. Reactivity to a 47 kDa band co-migrating with α-enolase specifically in *in vitro*-citrullinated lysates and not untreated lysates was detected in 17 out of 52 RA sera, all of which also contained ACPA [183]. Because in an histological analysis of RA synovial membranes some degree of co-localization was observed between the abundant cellular staining with an anti-α-enolase antibody and scant cellular staining by the 'anti-citrulline antibody', it was proposed that citrullinated α-enolase could be a synovial autoantigen and that it could drive the ACPA response [183]. However, further work is needed to substantiate this hypothesis. In particular, evaluation is required of the prevalence of antibodies specific for the citrullinated form of α-enolase in RA as well in non-RA sera, notably in sera from patients suffering from numerous other disease conditions where a reactivity to native α-enolase has been shown, together with an immunochemical confirmation of the presence of citrullinated α-enolase in RA synovial tissues. Nevertheless, it was also noted that the amino acid sequence of α-enolase is highly conserved and that considerable similarity exists with prokaryotic α-enolases. Because of the expression of an enzyme with a PAD activity in at least one bacterium, the oral pathogen *Porphyromonas gingivalis*, an intriguing speculation from that study was that antibodies to human citrullinated α-enolase (presumably a subset of

ACPA) could be produced during an anti-infectious immune response to bacterial α-enolase citrullinated by a bacterial PAD [183]. Moreover, given that *P. gingivalis* is an oral pathogen strongly implicated in the pathogenesis of adult periodontitis and that this disease and RA share some characteristics, such as the influence of HLA-DR*0401 and *0404 haplotypes of the *HLA-DRB1* gene, the attractiveness of the hypothesis that immune response to citrullinated products of the *P. gingivalis* PAD activity initiates the ACPA response, at least in a subset of patients, is enhanced [178].

16.8 Goals for future research

As we have seen, differences in the clinical course and both genetic and environmental influences between ACPA-positive and -negative RA have started to clearly emerge. Accordingly, and wisely, it has recently been proposed that from now on a distinction between samples or measurements originating from the two disease categories be systematically made in all clinical, pathophysiogical, genetic or therapeutic studies to enhance our capacity to distinguish possible differences in the pathophysiological pathways involved [178]. As far as ACPA-positive RA is concerned, numerous questions on the origin and on the mechanisms of the implication of autoimmunity to citrullinated proteins in the disease pathophysiology remain unanswered.

Some of our efforts should be devoted to a better understanding of how autoreactivity to citrullinated proteins plays a role in RA synovitis. In particular, based on their abundance in RA synovial joints and about the prevalence of their recognition by ACPA-positive sera, a clear vision should soon be possible of what citrullinated joint targets of ACPA are likely to substantially contribute in an injurious immune reaction. As inhibition of the activity or of the expression of the PADs responsible for the generation of these targets may constitute a valuable therapeutic strategy for RA, the cell(s) and mechanisms involved also should be more precisely characterized. Notably, investigations are necessary into the apoptosis and/or necrosis events of infiltrating or resident cells possibly leading to the release of PADs in the extracellular milieu, explaining how extracellular targets, such as fibrin, can become PAD substrates while PADs are intracellular enzymes with no secretory signal peptide. Moreover, further studies on the effector pathways triggered following the interaction of ACPA with their citrullinated synovial targets are needed that should elucidate the involvement of complement components and better characterize the determinants and consequences of FcγR-mediated responses. The recent availability of anti-B cell strategies in the clinical management of RA now permits studies of how clinical improvement and clinical relapse are paralleled by variations in the levels of ACPA production and in the presence of corresponding specific B cells in the blood as well as in solid tissues such as the synovial membrane or the bone marrow. This research should provide compelling evidence of the involvement of autoimmune reactions to citrullinated proteins in RA pathophysiology. Moreover, recent results encourage believing that animal models of RA based on an autoimmune response to citrullinated proteins will soon be available. These should prove useful in future pathophysiological researches and may also be valuable in the

assessment and validation of new therapeutic approaches. Another area of interest that should not be neglected is the identification and characterization of the so-far elusive T cells that may play a role in triggering the B cell response, as a gain in the knowledge of both what maintains and what initiates this immune response is to be expected from the identification of their (citrullinated or not citrullinated) antigen target.

To open the way towards even earlier disease-modifying therapeutic strategies, other efforts should aim at understanding what initiates the immune response to citrullinated proteins. This will involve not only epidemiological studies, preferably in genetically well characterized cohorts, but also bioclinical approaches to estimate the overlap between ACPA and antibody responses triggered by the suspected environmental factor, and the use of this factor in experimental approaches, *in vitro* and in animals, to try and reproduce autoimmunization and/or pathophysiological events. Such animal studies may help resolving the intriguing question as to how an autoimmune response that may have been initiated somewhere else in the body or that may target an ubiquitously expressed autoantigen can end up affecting predominantly the joints.

When some or all of this necessary progress has been made, a better understanding of how the autoimmune response to citrullinated proteins is generated and of the mechanisms by which it participates in RA pathophysiology is to be expected.

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References

1. Nienhuis, R.L.F. and Mandema, E. (1964) A new serum factor in patients with rheumatoid arthritis: the antiperinuclear factor. *Annals of the Rheumatic Diseases* **23**, 302–305.
2. Sebbag, M., Simon, M., Vincent, C. *et al.* (1995) The antiperinuclear factor and the so-called antikeratin antibodies are the same rheumatoid arthritis-specific autoantibodies. *Journal of Clinical Investigation* **95**, 2672–2679.
3. Young, B.J., Mallya, R.K., Leslie, R.D. *et al.* (1979) Anti-keratin antibodies in rheumatoid arthritis. *British Medical Journal* **2**, 97–99.
4. Girbal, E., Sebbag, M., Gomès-Daudrix, V. *et al.* (1993) Characterisation of the rat oesophagus epithelium antigens defined by the so-called 'antikeratin antibodies', specific for rheumatoid arthritis. *Annals of the Rheumatic Diseases* **52**, 749–757.
5. Simon, M., Girbal, E., Sebbag, M. *et al.* (1993) The cytokeratin filament-aggregating protein filaggrin is the target of the so-called "antikeratin antibodies," autoantibodies specific for rheumatoid arthritis. *Journal of Clinical Investigation* **92**, 1387–1393.
6. Chavanas, S., Méchin, M.C., Nachat, R. *et al.* (2006) Peptidylarginine deiminases and deimination in biology and pathology: relevance to skin homeostasis. *Journal of Dermatological Science* **44**, 63–72.

7. Méchin, M.C., Sebbag, M., Arnaud, J. *et al.* (2007) Update on peptidylarginine deiminases and deimination in skin physiology and severe human diseases. *International Journal of Cosmetic Science* **29**, 147–168.
8. Nachat, R., Méchin, M.C., Charveron, M. *et al.* (2005) Peptidylarginine deiminase isoforms are differentially expressed in the anagen hair follicles and other human skin appendages. *Journal of Investigative Dermatology* **125**, 34–41.
9. Nachat, R., Méchin, M.C., Takahara, H. *et al.* (2005) Peptidylarginine deiminase isoforms 1–3 are expressed in the epidermis and involved in the deimination of K1 and filaggrin. *Journal of Investigative Dermatology* **124**, 384–393.
10. Urano, Y., Watanabe, K., Sakaki, A. *et al.* (1990) Immunohistochemical demonstration of peptidylarginine deiminase in human sweat glands. *American Journal of Dermatopathology* **12**, 249–255.
11. Ishigami, A., Ohsawa, T., Asaga, H. *et al.* (2002) Human peptidylarginine deiminase type II: molecular cloning, gene organization, and expression in human skin. *Archives of Biochemistry and Biophysics* **407**, 25–31.
12. Sambandam, T., Belousova, M., Accaviti-Loper, M.A. *et al.* (2004) Increased peptidylarginine deiminase type II in hypoxic astrocytes. *Biochemical and Biophysical Research, Communications* **325**, 1324–1329.
13. Ishigami, A., Ohsawa, T., Hiratsuka, M. *et al.* (2005) Abnormal accumulation of citrullinated proteins catalyzed by peptidylarginine deiminase in hippocampal extracts from patients with Alzheimer's disease. *Journal of Neuroscience Research* **80**, 120–128.
14. Vossenaar, E.R., Radstake, T.R., van der Heijden, A. *et al.* (2004) Expression and activity of citrullinating peptidylarginine deiminase enzymes in monocytes and macrophages. *Annals of the Rheumatic Diseases* **63**, 373–381.
15. Kanno, T., Kawada, A., Yamanouchi, J. *et al.* (2000) Human peptidylarginine deiminase type III: molecular cloning and nucleotide sequence of the cDNA, properties of the recombinant enzyme, and immunohistochemical localization in human skin. *Journal of Investigative Dermatology* **115**, 813–823.
16. Méchin, M.C., Enji, M., Nachat, R. *et al.* (2005) The peptidylarginine deiminases expressed in human epidermis differ in their substrate specificities and subcellular locations. *Cellular and Molecular Life Sciences* **62**, 1984–1995.
17. Nakashima, K., Hagiwara, T., Ishigami, A. *et al.* (1999) Molecular characterization of peptidylarginine deiminase in HL-60 cells induced by retinoic acid and 1alpha,25-dihydroxyvitamin D(3). *Journal of Biological Chemistry* **274**, 27786–27792.
18. Asaga, H., Nakashima, K., Senshu, T. *et al.* (2001) Immunocytochemical localization of peptidylarginine deiminase in human eosinophils and neutrophils. *Journal of Leukocyte Biology* **70**, 46–51.
19. Chang, X., Yamada, R., Suzuki, A. *et al.* (2005) Localization of peptidylarginine deiminase 4 (PADI4) and citrullinated protein in synovial tissue of rheumatoid arthritis. *Rheumatology (Oxford)* **44**, 40–50.
20. Foulquier, C., Sebbag, M., Clavel, C. *et al.* (2007) Peptidyl arginine deiminase type 2 (PAD-2) and PAD-4 but not PAD-1, PAD-3, and PAD-6 are expressed in rheumatoid arthritis synovium in close association with tissue inflammation. *Arthritis and Rheumatism* **56**, 3541–3553.
21. Wright, P.W., Bolling, L.C., Calvert, M.E. *et al.* (2003) ePAD, an oocyte and early embryo-abundant peptidylarginine deiminase-like protein that localizes to egg cytoplasmic sheets. *Developmental Biology* **256**, 73–88.
22. Inagaki, M., Takahara, H., Nishi, Y. *et al.* (1989) Ca2 + -dependent deimination-induced disassembly of intermediate filaments involves specific modification of the amino-terminal head domain. *Journal of Biological Chemistry* **264**, 18119–18127.
23. Tarcsa, E., Marekov, L.N., Mei, G. *et al.* (1996) Protein unfolding by peptidylarginine deiminase. Substrate specificity and structural relationships of, the natural substrates trichohyalin and filaggrin. *Journal of Biological Chemistry* **271**, 30709–30716.
24. Gyorgy, B., Toth, E., Tarcsa, E. *et al.* (2006) Citrullination: a posttranslational modification in health and disease. *International Journal of Biochemistry & Cell, Biology* **38**, 1662–1677.

25. Senshu, T., Kan, S., Ogawa, H. *et al.* (1996) Preferential deimination of keratin K1 and filaggrin during the terminal differentiation of human epidermis. *Biochemical and Biophysical Research, Communications* **225**, 712–719.
26. Moscarello, M.A., Wood, D.D., Ackerley, C. and Boulias, C. (1994) Myelin in multiple sclerosis is developmentally immature. *Journal of Clinical Investigation* **94**, 146–154.
27. Harauz, G. and Musse, A.A. (2007) A tale of two citrullines–structural and functional aspects of myelin basic protein deimination in health and disease. *Neurochemical Research* **32**, 137–158.
28. Liu, M., Oh, A., Calarco, P. *et al.* (2005) Peptidylarginine deiminase (PAD) is a mouse cortical granule protein that plays a role liu. *Reproductive Biology and Endocrinology* **3**, 42.
29. Esposito, G., Vitale, A.M., Leijten, F.P. *et al.* (2007) Peptidylarginine deiminase (PAD) 6 is essential for oocyte cytoskeletal sheet formation and female fertility. *Molecular and Cellular Endocrinology* **273**, 25–31.
30. Cuthbert, G.L., Daujat, S., Snowden, A.W. *et al.* (2004) Histone deimination antagonizes arginine methylation. *Cell* **118**, 545–553.
31. Wang, Y., Wysocka, J., Sayegh, J. *et al.* (2004) Human PAD4 regulates histone arginine methylation levels via demethylimination. *Science* **306**, 279–283.
32. Raijmakers, R., Zendman, A.J., Egberts, W.V. *et al.* (2007) Methylation of arginine residues interferes with citrullination by peptidylarginine deiminases *in vitro*. *Journal of Molecular Biology* **367**, 1118–1129.
33. Girbal-Neuhauser, E., Durieux, J.J., Arnaud, M. *et al.* (1999) The epitopes targeted by the rheumatoid arthritis-associated antifilaggrin autoantibodies are posttranslationally generated on various sites of (pro)filaggrin by deimination of arginine residues. *Journal of Immunology* **162**, 585–594.
34. Schellekens, G.A., de Jong, B.A., van den Hoogen, F.H. *et al.* (1998) Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. *Journal of Clinical Investigation* **101**, 273–281.
35. Senshu, T., Akiyama, K., Kan, S. *et al.* (1995) Detection of deiminated proteins in rat skin: probing with a monospecific antibody after modification of citrulline residues. *Journal of Investigative Dermatology* **105**, 163–169.
36. Union, A., Meheus, L., Humbel, R.L. *et al.* (2002) Identification of citrullinated rheumatoid arthritis-specific epitopes in natural filaggrin relevant for antifilaggrin autoantibody detection by line immunoassay. *Arthritis and Rheumatism* **46**, 1185–1195.
37. Sondag-Tschroots, I.R., Aaij, C., Smit, J.W. and Feltkamp, T.E. (1979) The antiperinuclear factor. 1. The diagnostic significance of the antiperinuclear factor for rheumatoid arthritis. *Annals of the Rheumatic Diseases* **38**, 248–251.
38. Johnson, G.D., Carvalho, A., Holborow, E.J. *et al.* (1981) Antiperinuclear factor and keratin antibodies in rheumatoid arthritis. *Annals of the Rheumatic Diseases* **40**, 263–266.
39. Mallya, R.K., Young, B.J., Pepys, M.B. *et al.* (1983) Anti-keratin antibodies in rheumatoid arthritis: frequency and correlation with other features of the disease. *Clinical and Experimental Immunology* **51**, 17–20.
40. Ordeig, J. and Guardia, J. (1984) Diagnostic value of antikeratin antibodies in rheumatoid arthritis. *Journal of Rheumatology* **11**, 602–604.
41. Hajiroussou, V.J., Skingle, J., Gillett, A.P. and Webley, M. (1985) Significance of antikeratin antibodies in rheumatoid arthritis. *Journal of Rheumatology* **12**, 57–59.
42. Youinou, P., Le Goff, P., Colaco, C.B. *et al.* (1985) Antikeratin antibodies in serum and synovial fluid show specificity for rheumatoid arthritis in a study of connective tissue diseases. *Annals of the Rheumatic Diseases* **44**, 450–454.
43. Kirstein, H. and Mathiesen, F.K. (1987) Antikeratin antibodies in rheumatoid arthritis. Methods and clinical significance. *Scandinavian Journal of Rheumatology* **16**, 331–338.
44. Janssens, X., Veys, E.M., Verbruggen, G. and Declercq, L. (1988) The diagnostic significance of the antiperinuclear factor for rheumatoid arthritis. *Journal of Rheumatology* **15**, 1346–1350.

45. Vincent, C., Serre, G., Lapeyre, F. *et al.* (1989) High diagnostic value in rheumatoid arthritis of antibodies to the stratum corneum of rat oesophagus epithelium, so-called 'antikeratin antibodies'. *Annals of the Rheumatic Diseases* **48**, 712–722.
46. Youinou, P., Le Goff, P., Dumay, A. *et al.* (1990) The antiperinuclear factor. I. Clinical and serologic associations. *Clinical and Experimental Rheumatology* **8**, 259–264.
47. von Essen, R., Kurki, P., Isomaki, H. *et al.* (1993) Prospect for an additional laboratory criterion for rheumatoid arthritis. *Scandinavian Journal of Rheumatology* **22**, 267–272.
48. Vincent, C., Nogueira, L., Clavel, C. *et al.* (2005) Autoantibodies to citrullinated proteins: ACPA. *Autoimmunity* **38**, 17–24.
49. Schellekens, G.A., Visser, H., de Jong, B.A. *et al.* (2000) The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis and Rheumatism* **43**, 155–163.
50. Coenen, D., Verschueren, P., Westhovens, R. and Bossuyt, X. (2007) Technical and diagnostic performance of 6 assays for the measurement of citrullinated protein/peptide antibodies in the diagnosis of rheumatoid arthritis. *Clinical Chemistry* **53**, 498–504.
51. Masson-Bessière, C., Sebbag, M., Durieux, J.J. *et al.* (2000) In the rheumatoid pannus, anti-filaggrin autoantibodies are produced by local plasma cells and constitute a higher proportion of IgGs than in synovial fluid and serum. *Clinical and Experimental Immunology* **119**, 544–552.
52. Iwaki-Egawa, S., Matsuno, H., Ogawa, Y. and Watanabe, Y. (2005) Production of anti-CCP antibodies and matrix metalloproteinase-3 by human rheumatoid arthritis synovial tissues using SCID mice. *Annals of the Rheumatic Diseases* **64**, 1094–1095.
53. Masson-Bessière, C., Sebbag, M., Girbal-Neuhauser, E. *et al.* (2001) The major synovial targets of the rheumatoid arthritis-specific antifilaggrin autoantibodies are deiminated forms of the alpha- and beta-chains of fibrin. *Journal of Immunology* **166**, 4177–4184.
54. Chapuy-Regaud, S., Sebbag, M., Baeten, D. *et al.* (2005) Fibrin deimination in synovial tissue is not specific for rheumatoid arthritis but commonly occurs during synovitides. *Journal of Immunology* **174**, 5057–5064.
55. Edwards, J.C. and Cambridge, G. (2006) B-cell targeting in rheumatoid arthritis and other autoimmune diseases. *Nature Reviews Immunology* **6**, 394–403.
56. Petkova, S.B., Konstantinov, K.N., Sproule, T.J. *et al.* (2006) Human antibodies induce arthritis in mice deficient in the low-affinity inhibitory IgGs receptor Fc gamma RIIB. *Journal of Experimental Medicine* **203**, 275–280.
57. Paimela, L., Gripenberg, M., Kurki, P. and Leirisalo-Repo, M. (1992) Antikeratin antibodies: diagnostic and prognostic markers for early rheumatoid arthritis. *Annals of the Rheumatic Diseases* **51**, 743–746.
58. Berthelot, J.M., Maugars, Y., Castagne, A. *et al.* (1997) Antiperinuclear factors are present in polyarthritis before ACR criteria for rheumatoid arthritis are fulfilled. *Annals of the Rheumatic Diseases* **56**, 123–125.
59. Goldbach-Mansky, R., Lee, J., McCoy, A. *et al.* (2000) Rheumatoid arthritis associated autoantibodies in patients with synovitis of recent onset. *Arthritis Research* **2**, 236–243.
60. Paimela, L., Palosuo, T., Aho, K. *et al.* (2001) Association of autoantibodies to filaggrin with an active disease in early rheumatoid arthritis. *Annals of the Rheumatic Diseases* **60**, 32–35.
61. Jansen, A.L., van der Horst-Bruinsma, I., van Schaardenburg, D. *et al.* (2002) Rheumatoid factor and antibodies to cyclic citrullinated Peptide differentiate rheumatoid arthritis from undifferentiated polyarthritis in patients with early arthritis. *Journal of Rheumatology* **29**, 2074–2076.
62. van Gaalen, F.A., Linn-Rasker, S.P., van Venrooij, W.J. *et al.* (2004) Autoantibodies to cyclic citrullinated peptides predict progression to rheumatoid arthritis in patients with undifferentiated arthritis: a prospective cohort study. *Arthritis and Rheumatism* **50**, 709–715.
63. Kastbom, A., Strandberg, G., Lindroos, A. and Skogh, T. (2004) Anti-CCP antibody test predicts the disease course during 3 years in early rheumatoid arthritis (the Swedish TIRA project). *Annals of the Rheumatic Diseases* **63**, 1085–1089.

64. Nielen, M.M., van der Horst, A.R., van Schaardenburg, D. *et al.* (2005) Antibodies to citrullinated human fibrinogen (ACF) have diagnostic and prognostic value in early arthritis. *Annals of the Rheumatic Diseases* **64**, 1199–1204.
65. Raza, K., Breese, M., Nightingale, P. *et al.* (2005) Predictive value of antibodies to cyclic citrullinated peptide in patients with very early inflammatory arthritis. *Journal of Rheumatology* **32**, 231–238.
66. Quinn, M.A., Gough, A.K., Green, M.J. *et al.* (2006) Anti-CCP antibodies measured at disease onset help, identify seronegative rheumatoid arthritis and predict radiological and functional outcome. *Rheumatology (Oxford)* **45**, 478–480.
67. Vander Cruyssen, B., Cantaert, T., Nogueira, L. *et al.* (2006) Diagnostic value of anti-human citrullinated fibrinogen ELISA and comparison with four other anti-citrullinated protein assays. *Arthritis Research and Therapy* **8**, R122.
68. Machold, K.P., Stamm, T.A., Nell, V.P. *et al.* (2007) Very recent onset rheumatoid arthritis: clinical and, serological patient characteristics associated with radiographic progression over the first years of disease. *Rheumatology (Oxford)* **46**, 342–349.
69. Ates, A., Karaaslan, Y. and Aksaray, S. (2007) Predictive value of antibodies to cyclic citrullinated peptide in patients with early arthritis. *Clinical Rheumatology* **26**, 499–504.
70. Kurki, P., Aho, K., Palosuo, T. and Heliovaara, M. (1992) Immunopathology of rheumatoid arthritis. Antikeratin antibodies precede the clinical disease. *Arthritis and Rheumatism* **35**, 914–917.
71. Rantapaa-Dahlqvist, S., de Jong, B.A., Berglin, E. *et al.* (2003) Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis and Rheumatism* **48**, 2741–2749.
72. Nielen, M.M., van Schaardenburg, D., Reesink, H.W. *et al.* (2004) Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. *Arthritis and Rheumatism* **50**, 380–386.
73. Berglin, E., Padyukov, L., Sundin, U. *et al.* (2004) A combination of autoantibodies to cyclic citrullinated peptide (CCP) and HLA-DRB1 locus antigens is strongly associated with future onset of rheumatoid arthritis. *Arthritis Research and Therapy* **6**, R303–R308.
74. Meyer, O., Combe, B., Elias, A. *et al.* (1997) Autoantibodies predicting the outcome of rheumatoid arthritis: evaluation in two subsets of patients according to severity of radiographic damage. *Annals of the Rheumatic Diseases* **56**, 682–685.
75. Hayem, G., Chazerain, P., Combe, B. *et al.* (1999) Anti-Sa antibody is an accurate diagnostic and prognostic marker in adult rheumatoid arthritis. *Journal of Rheumatology* **26**, 7–13.
76. De Rycke, L., Peene, I., Hoffman, I.E.A. *et al.* (2004) Rheumatoid factor and anticitrullinated protein antibodies in rheumatoid arthritis: diagnostic value, associations with radiological progression rate, and extra-articular manifestations. *Annals of the Rheumatic Diseases* **63**, 1587–1593.
77. Bongi, S.M., Manetti, R., Melchiorre, D. *et al.* (2004) Anti-cyclic citrullinated peptide antibodies are highly associated with severe bone lesions in rheumatoid arthritis anti-CCP and bone damage in RA. *Autoimmunity* **37**, 495–501.
78. Samanci, N., Ozdem, S., Akbas, H. *et al.* (2005) Diagnostic value and clinical significance of anti-CCP in patients with advanced rheumatoid arthritis. *Journal of the National Medical Association* **97**, 1120–1126.
79. del Val del Amo, N., Ibanez Bosch, R., Fito Manteca, C. *et al.* (2006) Anti-cyclic citrullinated peptide antibody in rheumatoid arthritis: relation with disease aggressiveness. *Clinical and Experimental Rheumatology* **24**, 281–286.
80. Korkmaz, C., Us, T., Kasifoglu, T. and Akgun, Y. (2006) Anti-cyclic citrullinated peptide (CCP) antibodies in patients with long-standing rheumatoid arthritis and their relationship with extra-articular manifestations. *Clinical Biochemistry* **39**, 961–965.
81. Mewar, D., Coote, A., Moore, D.J. *et al.* (2006) Independent associations of anti-cyclic citrullinated peptide antibodies and rheumatoid factor with radiographic severity of rheumatoid arthritis. *Arthritis Research and Therapy* **8**, R128.
82. Agrawal, S., Misra, R. and Aggarwal, A. (2007) Autoantibodies in rheumatoid arthritis: association with severity of disease in established RA. *Clinical Rheumatology* **26**, 201–204.

83. Zhao, Y., Tian, X. and Li, Z. (2007) Prevalence and clinical significance of antibodies to citrullinated fibrinogen (ACF) in Chinese patients with rheumatoid arthritis. *Clinical Rheumatology* **26**, 1505–1512.
84. Munoz-Fernandez, S., Alvarez-Doforno, R., Gonzalez-Tarrio, J.M. *et al.* (1999) Antiperinuclear factor as a prognostic marker in rheumatoid arthritis. *Journal of Rheumatology* **26**, 2572–2577.
85. Kroot, E.J., de Jong, B.A., van Leeuwen, M.A. *et al.* (2000) The prognostic value of anti-cyclic citrullinated peptide antibody in patients with recent-onset rheumatoid arthritis. *Arthritis and Rheumatism* **43**, 1831–1835.
86. Forslin, K., Vincent, C., Serre, G. and Svensson, B. (2001) Antifilaggrin antibodies in early rheumatoid arthritis may predict radiological progression. *Scandinavian Journal of Rheumatology* **30**, 221–224.
87. Genevay, S., Hayem, G., Verpillat, P. and Meyer, O. (2002) An eight year prospective study of outcome prediction by antiperinuclear factor and antikeratin antibodies at onset of rheumatoid arthritis. *Annals of the Rheumatic Diseases* **61**, 734–736.
88. Meyer, O., Labarre, C., Dougados, M. *et al.* (2003) Anticitrullinated protein/peptide antibody assays in early rheumatoid arthritis for predicting five year radiographic damage. *Annals of the Rheumatic Diseases* **62**, 120–126.
89. Vencovsky, J., Machacek, S., Sedova, L. *et al.* (2003) Autoantibodies can be prognostic markers of an erosive disease in early rheumatoid arthritis. *Annals of the Rheumatic Diseases* **62**, 427–430.
90. Forslind, K., Ahlmen, M., Eberhardt, K. *et al.* (2004) Prediction of radiological outcome in early rheumatoid arthritis in clinical practice: role of antibodies to citrullinated peptides (anti-CCP). *Annals of the Rheumatic Diseases* **63**, 1090–1095.
91. Lindqvist, E., Eberhardt, K., Bendtzen, K. *et al.* (2005) Prognostic laboratory markers of joint damage in rheumatoid arthritis. *Annals of the Rheumatic Diseases* **64**, 196–201.
92. van der Helm-van Mil, A.H., Verpoort, K.N., Breedveld, F.C. *et al.* (2005) Antibodies to citrullinated proteins and differences in clinical progression of rheumatoid arthritis. *Arthritis Research and Therapy* **7**, R949–R958.
93. Berglin, E., Johansson, T., Sundin, U. *et al.* (2006) Radiological outcome in rheumatoid arthritis is predicted by presence of antibodies against cyclic citrullinated peptide before and at disease onset, and by IgA-RF at disease onset. *Annals of the Rheumatic Diseases* **65**, 453–458.
94. Meyer, O., Nicaise-Roland, P., Santos, M.D. *et al.* (2006) Serial determination of cyclic citrullinated peptide autoantibodies predicted five-year radiological outcomes in a prospective cohort of patients with early rheumatoid arthritis. *Arthritis Research and Therapy* **8**, R40.
95. Kaltenhauser, S., Pierer, M., Arnold, S. *et al.* (2007) Antibodies against cyclic citrullinated peptide are, associated with the DRB1 shared epitope and predict joint erosion in rheumatoid arthritis. *Rheumatology (Oxford)* **46**, 100–104.
96. Innala, L., Kokkonen, H., Eriksson, C. *et al.* (2008) Antibodies against mutated citrullinated vimentin are a better predictor of disease activity at 24 months in early rheumatoid arthritis than antibodies against cyclic citrullinated peptides. *Journal of Rheumatology* **35**, 1002–1008.
97. Syversen, S.W., Gaarder, P.I., Goll, G.L. *et al.* (2008) High anti-cyclic citrullinated peptide levels and an algorithm of four variables predict radiographic progression in patients with rheumatoid arthritis: results from a 10-year longitudinal study. *Annals of the Rheumatic Diseases* **67**, 212–217.
98. Lundberg, K., Nijenhuis, S., Vossenaar, E.R. *et al.* (2005) Citrullinated proteins have increased immunogenicity and arthritogenicity and their presence in arthritic joints correlates with disease severity. *Arthritis Research and Therapy* **7**, R458–R467.
99. Kuhn, K.A., Kulik, L., Tomooka, B. *et al.* (2006) Antibodies against citrullinated proteins enhance tissue injury in experimental autoimmune arthritis. *Journal of Clinical Investigation* **116**, 961–973.
100. Vossenaar, E.R., Nijenhuis, S., Helsen, M.M. *et al.* (2003) Citrullination of synovial proteins in murine models of rheumatoid arthritis. *Arthritis and Rheumatism* **48**, 2489–2500.
101. Vossenaar, E.R., van Boekel, M.A., van Venrooij, W.J. *et al.* (2004) Absence of citrulline-specific autoantibodies in animal models of autoimmunity. *Arthritis and Rheumatism* **50**, 2370–2372.

102. Hill, J.A., Bell, D.A., Brintnell, W. *et al.* (2008) Arthritis induced by posttranslationally modified (citrullinated) fibrinogen in DR4-IE transgenic mice. *Journal of Experimental Medicine* **205**, 967–979.
103. Vincent, C., Serre, G., Basile, J.P. *et al.* (1990) Subclass distribution of IgGs antibodies to the rat oesophagus stratum corneum (so-called anti-keratin antibodies) in rheumatoid arthritis. *Clinical and Experimental Immunology* **81**, 83–89.
104. Chapuy-Regaud, S., Nogueira, L., Clavel, C. *et al.* (2005) IgGs subclass distribution of the rheumatoid arthritis-specific autoantibodies to citrullinated fibrin. *Clinical and Experimental Immunology* **139**, 542–550.
105. Clavel, C., Nogueira, L., Laurent, L. *et al.* (2008) Induction of macrophage secretion of tumor necrosis factor alpha through Fcgamma receptor IIa engagement by rheumatoid arthritis-specific autoantibodies to citrullinated proteins complexed with fibrinogen. *Arthritis and Rheumatism* **58**, 678–688.
106. Feldmann, M., Brennan, F.M. and Maini, R.N. (1996) Role of cytokines in rheumatoid arthritis. *Annual Review of Immunology* **14**, 397–440.
107. Feldmann, M. and Maini, R.N. (2001) Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned? *Annual Review of Immunology* **19**, 163–196.
108. Boross, P. and Verbeek, J.S. (2006) The complex role of Fcgamma receptors in the pathology of arthritis. *Springer Seminars in Immunopathology* **28** 339–350.
109. Desprès, N., Boire, G., Lopez-Longo, F.J. and Ménard, H.A. (1994) The Sa system: a novel antigen-antibody system specific for rheumatoid arthritis. *Journal of Rheumatology* **21**, 1027–1033.
110. Vossenaar, E.R., Despres, N., Lapointe, E. *et al.* (2004) Rheumatoid arthritis specific anti-Sa antibodies target citrullinated vimentin. *Arthritis Research and Therapy* **6**, R142–R150.
111. Menard, H.A., Lapointe, E., Rochdi, M.D. and Zhou, Z.J. (2000) Insights into rheumatoid arthritis derived from the Sa immune system. *Arthritis Research* **2**, 429–432.
112. Nijenhuis, S., Zendman, A.J., Vossenaar, E.R. *et al.* (2004) Autoantibodies to citrullinated proteins in rheumatoid arthritis: clinical performance and biochemical aspects of an RA-specific marker. *Clinica Chimica Acta* **350**, 17–34.
113. Cantaert, T., De Rycke, L., Bongartz, T. *et al.* (2006) Citrullinated proteins in rheumatoid arthritis: crucial. . .but not sufficient! *Arthritis and Rheumatism* **54**, 3381–3389.
114. Asaga, H., Yamada, M. and Senshu, T. (1998) Selective deimination of vimentin in calcium ionophore-induced apoptosis of mouse peritoneal macrophages. *Biochemical and Biophysical Research, Communications* **243**, 641–646.
115. Bang, H., Egerer, K., Gauliard, A. *et al.* (2007) Mutation and citrullination modifies vimentin to a novel autoantigen for rheumatoid arthritis. *Arthritis and Rheumatism* **56**, 2503–2511.
116. Mathsson, L., Mullazehi, M., Wick, M.C. *et al.* (2008) Antibodies against citrullinated vimentin in rheumatoid arthritis: higher sensitivity and extended prognostic value concerning future radiographic progression as compared with antibodies against cyclic citrullinated peptides. *Arthritis and Rheumatism* **58**, 36–45.
117. Dejaco, C., Klotz, W., Larcher, H. *et al.* (2006) Diagnostic value of antibodies against a modified citrullinated vimentin in rheumatoid arthritis. *Arthritis Research and Therapy* **8**, R119.
118. Sghiri, R., Bouagina, E., Zaglaoui, H. *et al.* (2007) Diagnostic performances of anti-cyclic citrullinated peptide antibodies in rheumatoid arthritis. *Rheumatology International* **27**, 1125–1130.
119. Tilleman, K., Van Steendam, K., Cantaert, T. *et al.* (2008) Synovial detection and autoantibody reactivity of, processed citrullinated isoforms of vimentin in inflammatory arthritides. *Rheumatology (Oxford)* **47**, 597–604.
120. Suzuki, A., Yamada, R., Ohtake-Yamanaka, M. *et al.* (2005) Anti-citrullinated collagen type I antibody is a target of autoimmunity in rheumatoid arthritis. *Biochemical and Biophysical Research, Communications* **333**, 418–426.
121. Burkhardt, H., Sehnert, B., Bockermann, R. *et al.* (2005) Humoral immune response to citrullinated collagen type II determinants in early rheumatoid arthritis. *European Journal of Immunology* **35**, 1643–1652.
122. Koivula, M.K., Aman, S., Karjalainen, A. *et al.* (2005) Are there autoantibodies reacting against citrullinated peptides derived from type I and type II collagens in patients with rheumatoid arthritis? *Annals of the Rheumatic Diseases* **64**, 1443–1450.

123. Yoshida, M., Tsuji, M., Kurosaka, D. *et al.* (2006) Autoimmunity to citrullinated type II collagen in rheumatoid arthritis. *Modern Rheumatology* **16**, 276–281.
124. Koivula, M.K., Ramberg, J., Aman, S. *et al.* (2005) Sensitive immunoassays for the autoantibodies reacting against citrullinated carboxy-terminal telopeptides of type I and type II collagens in patients with rheumatoid arthritis. *Clinical Chemistry and Laboratory Medicine* **43**, 1400–1405.
125. Koivula, M.K., Aman, S., Alasaarela, E. *et al.* (2006) Inhibitory characteristics of citrullinated telopeptides, of type I and II collagens for autoantibody binding in patients with rheumatoid arthritis. *Rheumatology (Oxford)* **45**, 1364–1369.
126. Koivula, M.K., Heliovaara, M., Ramberg, J. *et al.* (2007) Autoantibodies binding to citrullinated telopeptide of type II collagen and to cyclic citrullinated peptides predict synergistically the development of seropositive rheumatoid arthritis. *Annals of the Rheumatic Diseases* **66**, 1450–1455.
127. Koivula, M.K., Savolainen, E., Kaipiainen-Seppanen, O. *et al.* (2008) Sensitivity and specificity of autoantibodies binding, to citrullinated carboxyterminal telopeptides of types I and II collagens in an early arthritis series. *Rheumatology (Oxford)* **47**, 656–659.
128. Matsuo, K., Xiang, Y., Nakamura, H. *et al.* (2006) Identification of novel citrullinated autoantigens of synovium in rheumatoid arthritis using a proteomic approach. *Arthritis Research and Therapy* **8**, R175.
129. Skriner, K., Adolph, K., Jungblut, P.R. and Burmester, G.R. (2006) Association of citrullinated proteins with synovial exosomes. *Arthritis and Rheumatism* **54**, 3809–3814.
130. Hill, J.A., Al-Bishri, J., Gladman, D.D. *et al.* (2006) Serum autoantibodies that bind citrullinated fibrinogen are frequently found in patients with rheumatoid arthritis. *Journal of Rheumatology* **33**, 2115–2119.
131. Sebbag, M., Moinard, N., Auger, I. *et al.* (2006) Epitopes of human fibrin recognized by the rheumatoid arthritis-specific autoantibodies to citrullinated proteins. *European Journal of Immunology* **36**, 2250–2263.
132. Weinberg, J.B., Pippen, A.M. and Greenberg, C.S. (1991) Extravascular fibrin formation and dissolution in synovial tissue of patients with osteoarthritis and rheumatoid arthritis. *Arthritis and Rheumatism* **34**, 996–1005.
133. Busso, N. and Hamilton, J.A. (2002) Extravascular coagulation and the plasminogen activator/plasmin system in rheumatoid arthritis. *Arthritis and Rheumatism* **46**, 2268–2279.
134. Suzuki, A., Yamada, R., Chang, X. *et al.* (2003) Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. *Nature Genetics* **34**, 395–402.
135. Nakayama-Hamada, M., Suzuki, A., Kubota, K. *et al.* (2005) Comparison of enzymatic properties between hPADI2 and hPADI4. *Biochemical and Biophysical Research, Communications* **327**, 192–200.
136. De Rycke, L., Nicholas, A.P., Cantaert, T. *et al.* (2005) Synovial intracellular citrullinated proteins colocalizing with peptidyl arginine deiminase as pathophysiologically relevant antigenic determinants of rheumatoid arthritis-specific humoral autoimmunity. *Arthritis and Rheumatism* **52**, 2323–2330.
137. Chang, X., Yamada, R., Suzuki, A. *et al.* (2005) Citrullination of fibronectin in rheumatoid arthritis, synovial tissue. *Rheumatology (Oxford)* **44**, 1374–1382.
138. Verpoort, K.N., Jol-van der Zijde, C.M., Papendrecht-van der Voort, E.A. *et al.* (2006) Isotype distribution of anti-cyclic citrullinated peptide antibodies in undifferentiated arthritis and rheumatoid arthritis reflects an ongoing immune response. *Arthritis and Rheumatism* **54**, 3799–3808.
139. Hida, S., Miura, N.N., Adachi, Y. and Ohno, N. (2004) Influence of arginine deimination on antigenicity of fibrinogen. *Journal of Autoimmunity* **23**, 141–150.
140. Duplan, V., Foulquier, C., Clavel, C. *et al.* (2006) In the rat, citrullinated autologous fibrinogen is immunogenic but the induced autoimmune response is not arthritogenic. *Clinical and Experimental Immunology* **145**, 502–512.
141. Rubin, B. and Matron, C. (2005) The mouse immune response to human fibrinogen reveals an autoimmune component against mouse fibrinogen. *Cellular Immunology* **233**, 41–52.

142. Vossenaar, E.R., Smeets, T.J., Kraan, M.C. *et al.* (2004) The presence of citrullinated proteins is not specific for rheumatoid synovial tissue. *Arthritis and Rheumatism* **50**, 3485–3494.
143. Makrygiannakis, D., af Klint, E., Lundberg, I.E. *et al.* (2006) Citrullination is an inflammation-dependent process. *Annals of the Rheumatic Diseases* **65**, 1219–1222.
144. Bongartz, T., Cantaert, T., Atkins, S.R. *et al.* (2006) Citrullination in extra-articular manifestations of, rheumatoid arthritis. *Rheumatology (Oxford)* **46**, 70–75.
145. Baeten, D., Peene, I., Union, A. *et al.* (2001) Specific presence of intracellular citrullinated proteins in rheumatoid arthritis synovium: relevance to antifilaggrin autoantibodies. *Arthritis and Rheumatism* **44**, 2255–2262.
146. Baeten, D., Kruithof, E., De Rycke, L. *et al.* (2004) Diagnostic classification of spondylarthropathy and rheumatoid arthritis by synovial histopathology: a prospective study in 154 consecutive patients. *Arthritis and Rheumatism* **50**, 2931–2941.
147. Stastny, P. (1978) Association of the B-cell alloantigen DRw4 with rheumatoid arthritis. *New England Journal of Medicine* **298**, 869–871.
148. Wordsworth, B.P., Lanchbury, J.S., Sakkas, L.I. *et al.* (1989) HLA-DR4 subtype frequencies in rheumatoid, arthritis indicate that DRB1 is the major susceptibility locus within the HLA class II region. *Proceedings of the National Academy of Sciences USA* **86**, 10049–10053.
149. Gregersen, P.K., Silver, J. and Winchester, R.J. (1987) The shared epitope hypothesis. An approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. *Arthritis and Rheumatism* **30**, 1205–1213.
150. Forslin, K., Vincent, C., Serre, G. and Svensson, B. (2000) Antifilaggrin autoantibodies in early rheumatoid arthritis. *Scandinavian Journal of Rheumatology* **29**, 320–322.
151. Bas, S., Perneger, T.V., Mikhnevitch, E. *et al.* (2000) Association of rheumatoid factors and antifilaggrin, antibodies with severity of erosions in rheumatoid arthritis. *Rheumatology (Oxford)* **39**, 1082–1088.
152. Auger, I., Sebbag, M., Vincent, C. *et al.* (2005) Influence of HLA-DR genes on the production of rheumatoid arthritis-specific autoantibodies to citrullinated fibrinogen. *Arthritis and Rheumatism* **52**, 3424–3432.
153. Huizinga, T.W., Amos, C.I., van der Helm-van Mil, A.H. *et al.* (2005) Refining the complex rheumatoid arthritis phenotype based on specificity of the HLA-DRB1 shared epitope for antibodies to citrullinated proteins. *Arthritis and Rheumatism* **52**, 3433–3438.
154. van der Helm-van Mil, A.H., Verpoort, K.N., Breedveld, F.C. *et al.* (2006) The HLA-DRB1 shared epitope alleles are primarily a risk factor for anti-cyclic citrullinated peptide antibodies and are not an independent risk factor for development of rheumatoid arthritis. *Arthritis and Rheumatism* **54**, 1117–1121.
155. Klareskog, L., Stolt, P., Lundberg, K. *et al.* (2006) A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination. *Arthritis and Rheumatism* **54**, 38–46.
156. Hill, J.A., Southwood, S., Sette, A. *et al.* (2003) Cutting edge: the conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. *Journal of Immunology* **171**, 538–541.
157. Ireland, J., Herzog, J. and Unanue, E.R. (2006) Cutting edge: unique T cells that recognize citrullinated peptides are a feature of protein immunization. *Journal of Immunology* **177**, 1421–1425.
158. Vang, T., Miletic, A.V., Arimura, Y. *et al.* (2008) Protein tyrosine phosphatases in autoimmunity. *Annual Review of Immunology* **26**, 29–55.
159. Wesoly, J., van der Helm-van Mil, A.H., Toes, R.E. *et al.* (2005) Association of the PTPN22 C1858T single-nucleotide polymorphism with rheumatoid arthritis phenotypes in an inception cohort. *Arthritis and Rheumatism* **52**, 2948–2950.
160. Johansson, M., Arlestig, L., Hallmans, G. and Rantapaa-Dahlqvist, S. (2006) PTPN22 polymorphism and anti-cyclic citrullinated peptide antibodies in combination strongly predicts future onset of rheumatoid arthritis and has a specificity of 100% for the disease. *Arthritis Research and Therapy* **8**, R19.

161. Kokkonen, H., Johansson, M., Innala, L. *et al.* (2007) The PTPN22 1858C/T polymorphism is associated with anti-cyclic citrullinated peptide antibody-positive early rheumatoid arthritis in northern Sweden. *Arthritis Research and Therapy* **9**, R56.
162. Karlson, E.W., Chibnik, L.B., Cui, J. *et al.* (2008) Associations between human leukocyte antigen, PTPN22, CTLA4 genotypes and rheumatoid arthritis phenotypes of autoantibody status, age at diagnosis and erosions in a large cohort study. *Annals of the Rheumatic Diseases* **67**, 358–363.
163. Ikari, K., Kuwahara, M., Nakamura, T. *et al.* (2005) Association between PADI4 and rheumatoid arthritis: a replication study. *Arthritis and Rheumatism* **52**, 3054–3057.
164. Kang, C.P., Lee, H.S., Ju, H. *et al.* (2006) A functional haplotype of the PADI4 gene associated with increased rheumatoid arthritis susceptibility in Koreans. *Arthritis and Rheumatism* **54**, 90–96.
165. Plenge, R.M., Padyukov, L., Remmers, E.F. *et al.* (2005) Replication of putative candidate-gene associations with rheumatoid arthritis in >4,000 samples from North America and Sweden: association of susceptibility with PTPN22, CTLA4, and PADI4. *American Journal of Human Genetics* **77**, 1044–1060.
166. Hoppe, B., Haupl, T., Gruber, R. *et al.* (2006) Detailed analysis of the variability of peptidylarginine deiminase type 4 in German patients with rheumatoid arthritis: a case-control study. *Arthritis Research and Therapy* **8**, R34.
167. Barton, A., Bowes, J., Eyre, S. *et al.* (2004) A functional haplotype of the PADI4 gene associated with rheumatoid arthritis in a Japanese population is not associated in a United Kingdom population. *Arthritis and Rheumatism* **50**, 1117–1121.
168. Harney, S.M., Meisel, C., Sims, A.M. *et al.* (2005) Genetic and genomic studies of PADI4 in, rheumatoid arthritis. *Rheumatology (Oxford)* **44**, 869–872.
169. Martinez, A., Valdivia, A., Pascual-Salcedo, D. *et al.* (2005) PADI4 polymorphisms are not associated with, rheumatoid arthritis in the Spanish population. *Rheumatology (Oxford)* **44**, 1263–1266.
170. Caponi, L., Petit-Teixeira, E., Sebbag, M. *et al.* (2005) A family based study shows no association between rheumatoid arthritis and the PADI4 gene in a white French population. *Annals of the Rheumatic Diseases* **64**, 587–593.
171. Suzuki, M., Miyagi, J., Kuribayashi, M. *et al.* (2006) Evaluation of allele frequencies in the PADI4 gene and anti-cyclic citrullinated peptide antibodies of patients with rheumatoid arthritis in a Japanese population. *Annals of the Rheumatic Diseases* **65**, 1399–1400.
172. Cha, S., Choi, C.B., Han, T.U. *et al.* (2007) Association of anti-cyclic citrullinated peptide antibody levels with PADI4 haplotypes in early rheumatoid arthritis and with shared epitope alleles in very late rheumatoid arthritis. *Arthritis and Rheumatism* **56**, 1454–1463.
173. Oliver, J.E. and Silman, A.J. (2006) Risk factors for the development of rheumatoid arthritis. *Scandinavian Journal of Rheumatology* **35**, 169–174.
174. van der Helm-van Mil, A.H., Verpoort, K.N., le Cessie, S. *et al.* (2007) The HLA-DRB1 shared epitope alleles differ in the interaction with smoking and predisposition to antibodies to cyclic citrullinated peptide. *Arthritis and Rheumatism* **56**, 425–432.
175. Pedersen, M., Jacobsen, S., Garred, P. *et al.* (2007) Strong combined gene-environment effects in anti-cyclic citrullinated peptide-positive rheumatoid arthritis: a nationwide case-control study in Denmark. *Arthritis and Rheumatism* **56**, 1446–1453.
176. Lee, H.S., Irigoyen, P., Kern, M. *et al.* (2007) Interaction between smoking, the shared epitope, and anti-cyclic citrullinated peptide: a mixed picture in three large North American rheumatoid arthritis cohorts. *Arthritis and Rheumatism* **56**, 1745–1753.
177. Mikuls, T.R., Hughes, L.B., Westfall, A.O. *et al.* (2008) Cigarette smoking, disease severity, and autoantibody expression in African Americans with recent-onset rheumatoid arthritis. *Annals of the Rheumatic Diseases* **67**, 1529–1534.
178. Klareskog, L., Ronnelid, J., Lundberg, K. *et al.* (2008) Immunity to citrullinated proteins in rheumatoid arthritis. *Annual Review of Immunology* **26**, 651–675.
179. Makrygiannakis, D., Hermansson, M., Ulfgren, A.K. *et al.* (2008) Smoking increases peptidylarginine deiminase 2 enzyme expression in human lungs and increases citrullination in BAL cells. *Annals of the Rheumatic Diseases* **67**, 1488–1492.

180. Balandraud, N., Roudier, J. and Roudier, C. (2004) Epstein-Barr virus and rheumatoid arthritis. *Autoimmunity Reviews* **3**, 362–367.
181. Anzilotti, C., Merlini, G., Pratesi, F. *et al.* (2006) Antibodies to viral citrullinated peptide in rheumatoid arthritis. *Journal of Rheumatology* **33**, 647–651.
182. Pratesi, F., Tommasi, C., Anzilotti, C. *et al.* (2006) Deiminated Epstein-Barr virus nuclear antigen 1 is a target of anti-citrullinated protein antibodies in rheumatoid arthritis. *Arthritis and Rheumatism* **54**, 733–741.
183. Kinloch, A., Tatzer, V., Wait, R. *et al.* (2005) Identification of citrullinated alpha-enolase as a candidate autoantigen in rheumatoid arthritis. *Arthritis Research and Therapy* **7**, R1421–R1429.

Abstract

Deimination (citrullination) corresponds to the post-translational conversion of arginyl residues of peptides into citrullyl residues. This modification is essential in the formation of the epitopes recognized by autoantibodies to citrullinated proteins (ACPA), which are IgGs specifically found in the serum of patients with rheumatoid arthritis (RA), a frequent autoimmune disease associated with chronic and destructive inflammation of synovial joints. Here we make an account of the research that led from the initially described RA-associated anti-perinuclear factor and anti-keratin antibodies to ACPA, and to the recognition that B cell autoreactivity to citrullinated proteins occurs in the synovial membrane and probably plays a major role in RA synovitis. Moreover, the nature of the antigen targeted by ACPA in the RA synovium and the stimuli at play in the induction of the autoimmune response to citrullinated proteins are discussed.

Key words: rheumatoid arthritis; synovial membrane; autoantibodies; HLA-DRB1; peptidylarginine deiminase; ACPA; anti-CCP; citrulline; fibrin; filaggrin.

17

Hormones: epigenetic contributors to gender-biased autoimmunity

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17.1 Introduction

The ability of the immune system to distinguish self from non-self is essential to maintain non-responsiveness to self. While self-recognition occurs during early development, checkpoints that operate in the adult are critical for maintaining and establishing tolerance to self-antigens that appear after maturity [1] and self-tolerance continues in adult life. In autoimmune diseases it has been postulated that self-reactive cells that express low-affinity antigen receptors escape T cell clonal deletion [2]. Moreover, somatic mutations in adult life can potentially generate B cells with autoreactive antigen receptors. It is also well-established that loss of self-recognition and autoimmune disease are more prevalent in females than males [3]. For example, systemic lupus erythematosus (SLE) is a prototypic autoimmune disease that occurs nine times more often in women of reproductive age than men [3]. Females in general are more susceptible to autoimmunity and several diseases show strong female predominance (Table 17.1).

17.2 Oestrogen receptors

The nuclear receptor gene family includes receptors for oestrogens, androgens, glucocorticoids, thyroid hormones, retinoids and vitamin D_3 [4]. In this chapter we

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Table 17.1 Biased autoimmune diseases. Shown are some of the more common autoimmune diseases with a higher incidence in females compared with males. The list is not all-inclusive. Women are at greater risk (2.7 times) in general than men from developing autoimmune diseases. Based on [3].

Disease	Female preponderance (%)
Addison's disease	92
Chronic active hepatitis	88
Primary biliary cirrhosis	88
Scleroderma	92
Sjögren's syndrome	93
Systemic lupus erythematosus	88
Thyroiditis	94

focus on the female sex hormone oestrogen (oestradiol 17-β) as a potential contributor to the development of biased autoimmune disease. Oestrogen serves as a ligand for two specific receptor proteins termed oestrogen receptor-α (ER-α) and oestrogen receptor-β (ER-β). Oestrogen receptors (ERs) are transcription factors that bind to specific DNA sequences of target genes and alter transcription rates [5]. ER-α and ER-β bind oestradiol with equal affinity and both receptor subtypes interact with the oestrogen-response element (ERE), an inverted-repeat DNA sequence found in the promoter regions of many oestrogen-responsive genes [5]. Dimerization of the ER is required for transcriptional activity and in cells that express both receptor subtypes ER-α and ER-β can form heterodimers [6]. Recent data demonstrate that significant differences exist in gene-expression patterns controlled by ER-α and ER-β [4–6]. We have shown that both ER-α and ER-β are expressed in SLE and normal human T cells [7]. Furthermore, both receptor subtypes are functional in SLE T cells since ligand-selective agonists stimulate expression of two T cell activation markers, CD154 and calcineurin, respectively [8].

17.3 Oestrogen and autoimmunity

Although controversy about the role of sex hormones in moderating autoimmunity exists, flares of SLE may be associated with certain phases of the menstrual cycle, and occur more frequently during pregnancy when sex steroid levels in circulation are high [9]. Ovulation induction and *in vitro* fertilization procedures increase circulating oestradiol levels, which are associated with *de novo* SLE and flares [10]. After menopause, flares of lupus tend to subside and the number of new cases substantially decreases [11]. The safety of oral contraceptives and hormone-replacement therapy for women with stable or moderate SLE disease has been reported, in the USA, by the Safety of Estrogens in Lupus Erythematosus-National Assessment [12] and by a similar study completed in Mexico [13]. The results from those trials suggest that patients with

inactive disease can take oral contraceptives without increased risk of flares over the 1-year study period. More recently, however, a study from South Africa has raised a cautionary follow-up [14]. It is important to consider that in addition to age and dose-dependent evaluation, the duration of treatment could be a critical factor for women taking exogenous oestrogens in the form of hormone-replacement therapy [15]. The safety of oral contraceptives in SLE patients with active disease has not been studied.

Since oestrogen is a key regulator of molecules involved in inflammation, and circulating oestradiol is highest during the peak time of SLE onset, oestradiol could contribute to the development, progression or severity of SLE [16,17]. Oestrogen stimulates T cell activation markers [18,19] and regulates molecules that promote inflammation (reviewed in [20]). In animal models of SLE, female mice develop an earlier aggressive disease that is ameliorated by ovariectomy and/or androgen therapy [21]. Non-lupus-prone normal mice, engineered to express a heavy-chain anti-DNA antibody transgene can be induced into a lupus phenotype by oestrogen administration [22]. Oestrogen increases the production of cytokines [23] and immunoglobulins in circulation [24]. Oestrogen is reported to enhance the proliferation of T cells [25] and macrophages [26] and to directly stimulate the expression of genes in mouse B cells that allows some autoreactive cells to escape apoptosis [27]. Abnormal T cell regulation of B cells could result in antibody/autoantibody secretion [28]. SLE is the autoimmune disease with the largest number of detectable autoantibody specificities [29]. Therefore, oestrogen-dependent T cell stimulation provides a molecular link between hormone activation of the T cell, increased T cell–B cell interactions and SLE pathogenesis.

Oestrogen-responsive cells in the immune system include monocytes [30] and T cells in peripheral blood [31,32], thymus [33] and synovial tissue [30]. Peripheral blood mononuclear cells from females and males [7] express transcripts for ER-α and ER-β. Further analysis of ER-α transcripts in our laboratory [34] revealed that peripheral blood T cells, monocytes and B cell lines obtained from patients with SLE express ER-α transcripts similar to those from the same cell types of normal individuals. These findings have been confirmed by Kassi *et al.* [35], who reported that peripheral blood mononuclear cells from SLE patients express wild-type ER-α, ER-β and the same ER-α variants as normal individuals. The binding affinity, activity and quantity of ligand bound to ER-α are not altered in women with SLE [31,36]. Primary transcripts from both receptor subtypes are alternatively spliced, suggesting that differential sensitivity to ER action could occur by the predominance of an alternatively spliced variant in SLE T cells. Our data [34] do not support the postulate that any alternatively spliced ER-α variant is preferentially associated with SLE. However, other studies suggest that ER-α polymorphisms are associated with early [37] and late [38] SLE disease onset and with certain clinical patterns of disease. It remains to be shown that the alternatively spliced transcripts are translated into functional proteins and how these variants might contribute to SLE onset or disease progression.

The identification of both ER-α and ER-β transcripts in human T cells complicates elucidation of the potential mechanisms underlying ER action because altered responses could arise by changes in the action of either ER-α or ER-β. It is not known whether ERs regulate the same genes in T cells, or whether ER-β can inhibit ER-α-directed

responses [39]. The interaction of ERs is more complicated than simple receptor binding to EREs because ER can also bind with other transcription factors, such as activator protein 1 (AP-1) [40], resulting in productive multiprotein transcriptional regulatory complexes. Thus, it is possible that ER action is altered in SLE T cells because one receptor dominates, or because the ratio of receptor subtypes is different in SLE compared with normal T cells.

Increased levels of oestradiol in circulation would enhance oestrogenic effects on the immune system. In general, however, levels of oestradiol are within the normal range in women without and with SLE, although increased plasma oestradiol has been reported in some SLE patients [41]. The majority of evidence to date does not support increased levels of plasma oestradiol as a causative factor for the oestrogen-dependent effects reported in SLE.

The efficacy of oestrogen action in target cells is also dependent on the metabolism and turnover of the ligand–receptor complex. Oestrogen metabolism in some SLE patients is disordered, with a skewing towards biologically active metabolites [42]. Specifically, the hydroxylation of oestrone is favoured such that the 16-hydroxylated compounds are more abundant metabolites than the catechol oestrogens. The consequence of this altered metabolism could have important consequences since biologically active metabolites may prolong oestrogen action on target tissues. Oestriol is elevated in women with SLE and men with Klinefelter's syndrome, while patients of both sexes have increased amounts of 16-α hydroxyestrone. As mentioned earlier, most studies have not found significant differences in the levels of oestrogen in circulation in the form of oestradiol 17-β. Altered metabolism may contribute to increased oestrogen sensitivity in some patients. However, SLE T cells in culture respond with increased sensitivity to the poorly metabolized 2-fluoro-oestradiol [18], suggesting that altered metabolism is not the only mechanism responsible for the differential action of oestrogen in SLE T cells.

SLE is influenced by genetic factors and progress has been made in the identification of susceptibility genes [43,44]. Although multiple genes are involved, disease onset may not occur in the absence of environmental or exogenous triggers [45]. The concordance for SLE among monozygotic twins is relatively low [46]. Together, these observations suggest that factors in addition to genetic susceptibility contribute to the development of SLE. Furthermore, the molecular basis underlying deregulation of immune cells in SLE would appear to be global in nature since multiple genes are inappropriately regulated [47]. For example, measurement of 160 variants in the sera of SLE patients using a high-throughput protein microarray revealed 30 abnormally regulated proteins including cytokines, chemokines, growth factors and soluble receptors in SLE patients compared with control samples [48]. The question, for which there is no current answer, is what sort of molecular mechanisms could explain the deregulation of such a wide variety of different factors?

17.4 Foxp3 and ERs

One possibility that comes to mind regarding a global regulatory mechanism revolves around the concept that oestrogen, acting through the ER, could control a so-called

master regulatory gene such as Foxp3. Foxp3 is a member of the forkhead (Fox) gene family of winged helix transcription factors that binds to the promoter regions of well-characterized genes involved in T-cell activation [49,50]. The major role of Foxp3 is to dampen the induction of key genes upon T regulatory cell stimulation [50,51] and failure of Foxp3 suppression leads to over-expression of genes associated with autoimmune diseases. Patients with active SLE show decreased Foxp3 expression [52]. Oestrogen increases Foxp3 expression in mouse T regulatory cells ($CD4^+CD25^+$) [53,54]. Recent evidence suggests that Foxp3 must be stabilized by epigenetic modification for a permanent suppressor cell lineage to develop [55], opening a new and important line of investigation. We are just beginning to test whether Foxp3 is an ER target in human T cells.

17.5 ERs and histone modifications

In this review we focus on epigenetic changes that may be exerted by the more recently discovered contributor to transcriptional regulation known as the histone code [56,57]. Although less is known about the relevance of histone changes as relates to disease processes, evidence is beginning to accumulate that nuclear receptors will be key effectors of epigenetic change because they recruit proteins into complexes that modify histones and chromatin structure [58,59]. Chromatin, which serves as the template for eukaryotic genetic information, is comprised of histone and non-histone proteins that compact DNA in the nucleus. DNA is compacted by wrapping around nucleosomes which contain two molecules each of H2A, H2B, H3 and H4 small basic proteins that have charged N-termini protruding from the nucleosome core, referred to as histone tails. A major problem for any transcriptional regulatory protein is attaining access to DNA regulatory sites since most of the genome is highly compacted in a eukaryotic nucleus. Modification of the histone tail domains through acetylation, methylation, phosphorylation and other post-translational changes contributes to different epigenetic states [56]. For example, histone methylation regulates chromatin structure and depending on the site of methylation transcription can be activated or repressed [58]. ERs function as transcription factors and regulate target gene mRNA levels. They contain subdomains that are necessary for transcriptional activation because these regulatory domains form a docking surface for other proteins known as co-activators and co-repressors [60]. Nuclear receptor co-activators enhance transcription while nuclear co-repressors suppress transcription by modifying the histones at target gene sites.

17.6 The histone code

The histone code revolves around the concept that covalent modification of histones marks chromatin in a stable and heritable fashion that can lead to differences in ‘on’ and ‘off’ states of transcriptional activity [57]. Co-activators recruit enzymes that phosphorylate, acetylate and methylate histones and thereby modify chromatin

structure [60,61]. Histone acetylation has been known for some time to 'open' chromatin and allow access of transcription factors; however, not all forms of acetylation stimulate transcription and not all forms of deacetylation inhibit the process. Histone modifications do alter chromatin compaction but they also provide a code for additional regulatory events that lead to nucleosome phasing, assembly of the protein complex required for transcription initiation, protein recruitment or blockade from the chromatin template and regulation of a series of highly ordered but dynamic changes during transcription [60–63].

17.7 Co-activators

The development of microarray technology has been used to assess a more global view of the genome and, thereby, has enhanced our understanding of transcriptional regulation. It is clear that gene regulation is not achieved by the binding of a single factor; rather, there is a cyclic recruitment of factors, modification of chromatin and turnover of transcription complexes [62]. This allows for constant sensing of a variety of signalling inputs to finely control transcription [63]. The use of chromatin immunoprecipitation (ChIP) assays revealed that association of the ER to the promoter region of target genes stimulates the recruitment of numerous proteins, including the cAMP-response-element-binding protein (CREB), p300/CBP-associated factor, the co-activator-associated arginine methyltransferase 1, p160 co-activator proteins and RNA polymerase II [64–66]. Concomitant with this co-factor recruitment are changes in histone acetylation and relaxation of chromatin at the regions of transcriptional activation presumably facilitating the binding of transcription factors into multiprotein regulatory complexes. ChIP microarray analysis, also called ChIP-chip, of ER-binding sites on a chromosome-wide scale [66,67] revealed that most of the putative ER-binding sites were some distance from gene targets. The results were consistent with a model suggesting that distal chromatin regions function by looping out the DNA to bring the enhancer and promoter regions together.

17.8 Pioneer factors

Gene transcription is controlled by DNA-bound transcription factors that recruit co-factors involved in chromatin remodelling [60]. The recruitment of RNA polymerase II and TATA-box-binding proteins is a prerequisite but does not appear to be sufficient for transcriptional activation. In some instances, factors termed pioneer factors are associated with heterochromatic regions [67,68]. Pioneer factors serve to recruit transcriptional regulators such as the ER to specific DNA-binding sites where the ER recruits additional regulatory factors. Some of these factors modify histone proteins to open or unwind compacted chromatin and facilitate transcription [58,65]. A balance of co-activators and co-repressors is central to the magnitude and nature of a response to nuclear receptors and their ligands [69,70]. Moreover, the promoter context and

regulatory environment exert control over the histone modifiers and direct the activation or repression of transcription [5,69,70].

17.9 Co-repressors

The co-repressors repress gene transcription by blocking access of other factors to DNA regulatory regions. Two well-characterized co-repressors, silencing mediator of retinoid and thyroid hormone receptors (SMRT) and nuclear hormone receptor-corepressor (N-CoR), operate by recruiting histone deacetylases (HDACs) that deacetylate histones and interfere with transcriptional initiation [71,72]. It is important to note that co-repressors and co-activators function widely in transcriptional regulation. For example, p300 serves as a co-activator for nuclear factor κB [73] and it is also involved in transcriptional repression by nuclear factor κB [74]. Recent evidence suggests that the recruitment of co-activators and co-repressors to the promoter of ER target genes can be affected by the binding of oestrogen (ligand), antioestrogens and the ERE along the DNA [75,76]. In the absence of hormone, several of the nuclear receptors including the ER are unable to bind co-repressors. However, in the presence of hormone antagonists the ER undergoes a conformational change that facilitates co-repressor binding. The balance between activators and repressors may be a key concept for understanding ER-regulated gene expression.

17.10 ERs and cell proliferation

It is well established that ERs stimulate the proliferation of a variety of cell types including cells belonging to the immune system. Overexpression of cell-cycle-regulatory proteins may lead to abnormal oestrogen-dependent gene regulation. In breast cancer cells, cyclin D1 protein is often upregulated. It can function as a bridge and recruit steroid receptor co-activators to the ER and stimulate transcriptional activation in the absence of oestrogen [77]. The binding of cyclin D1 to the ER-α promoter increases expression because BRCA1, a repressor of ER-α transcription, is unable to bind when the promoter is occupied by cyclin D1 [78]. Altered signal transduction in SLE T cells results in abnormal levels of transcription factors and cell-adhesion molecules [79]. It remains to be established whether alterations in the cellular milieu and transcriptional regulatory proteins leads to abnormal ER regulation of target genes in SLE T cells.

17.11 Epigenetic changes in disease

Phenotypic plasticity and epigenetic changes may be central for understanding genetic disorders and other types of diseases including cancers [80,81]. It is becoming clear that epigenetic changes occur in stressed cells and that specific remodelling of chromatin

may be related to the type of stress [82]. Epigenetic changes are thought to underlie the development of some types of breast cancer and targeting of the factors involved to develop more specific therapies is a vigorous area of research [83]. In ER-negative breast cancer cells the promoter region is hypermethylated and ER mRNA is absent [84]. Treatment of ER-α negative cells with DNA methyltransferase or HDAC inhibitors lead to a resumption of ER mRNA expression and the production of a functional protein. The activation of ER gene expression using 5-aza-2′-deoxycytidine stimulates the release of a repressor complex that contains methyl-binding proteins [84].

17.12 ERs and SLE

ER-α is an epigenetically regulated gene [84] and T cells from SLE patients have decreased total genomic methylation compared with age-matched controls [85]. Decreased methylation of ER-α would be expected to increase transcript levels. Consistent with this possibility, a recent report [86] suggests that ER-α transcripts are higher in circulating T cells of SLE patients compared with normal T cells. At the protein level, however, we find that ER-α but not ER-β is lower in the T cells of some SLE patients compared with the amount in T cells from normal individuals (Figure 17.1 [8]). We do not know whether the level of mRNA corresponds with a lower level of ER-α protein in SLE T cells and this possibility is currently under investigation. It is important to clarify this issue because identification of the underlying mechanism will provide greater insight into ER effects in SLE.

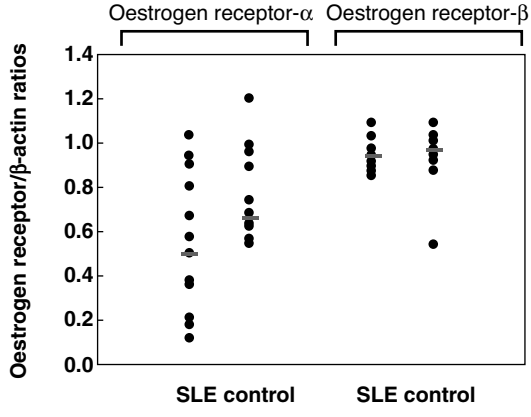

Figure 17.1 Measurement of oestrogen receptors in human T cells shows greater variation in the amount of ER-α in SLE compared with normal T cells. Data shown are the amount of ER-α and ER-β in T cell extracts, expressed as a ratio to the amount of β-actin in the same samples. Horizontal lines are the median values from 13 and 16 SLE patients and 13 and 11 normal controls for ER-α and ER-β, respectively. From [8]. Reproduced by permission of the *Journal of Rheumatology*.

ER-α is lost in a breast cancer cell line by direct binding of the transcription factor, Snail, with DNA-regulatory regions along the ER-α promoter [87]. Loss of ER-α signalling lead to altered transforming growth factor β signalling in that breast cancer cell line. Since transforming growth factor β is central to the development of tolerance [88] it is important to uncover the molecular basis for low ER-α protein in the T cells from some SLE patients and investigate how low ER-α contributes to SLE. Alternatively, low ER-α levels in SLE T cells could result from increased turnover of the protein. Protein degradation via the ubiquitin-proteasome system has emerged as a major regulator of nuclear receptor transcription [89,90]. The 20 S proteasome β subunit low molecular mass polypeptide 2 (LMP2) is necessary for ER-dependent transcription and cell-cycle progression [91]. A splice variant of ER-β, which has a unique C-terminus, antagonizes ER-α transactivation by stimulating proteasome degradation of ER-α [92]. While purely speculative at this point, inappropriate assembly and turnover of the ER-α complex could underpin the aberrant oestrogen-dependent gene regulation that we have reported [18,19].

17.13 Co-activators and phosphorylation

ER-mediated gene transcription is tissue and cell-specific and can be coordinately regulated by non-genomic signalling [93]. Oestrogen induces mitogen-activated protein kinase signalling, which is essential for cell proliferation. Regulation of nuclear co-factors by phosphorylation has been less well studied but it is a potential mechanism to impart specificity and selectivity to transcriptional regulation. A co-activator complex formed between helicases and p160/p300 co-activators binds more strongly to phosphorylated than non-phosphorylated ER-α [94]. Co-factor phosphorylation can activate or repress transcription and different kinase signalling pathways may exert differential effects [95].

SLE T cells are known to be deficient in protein phosphorylation [96], which could contribute to global deregulation of gene expression. Recent results from our laboratory [97] suggest that the T cells from SLE patients with inactive or mild disease respond to oestradiol by suppressing phosphorylation but as disease activity increases the suppressive effect is lost. This result was surprising since we anticipated increased phosphorylation in response to oestradiol based on the results obtained in other cell types. Although the sample size in our study is relatively small, the results are important because they highlight the problems with oestrogen-dependent signalling and gene regulation in SLE T cells at many different levels. Although we have investigated numerous possibilities to identify the molecular basis for increased sensitivity of SLE T cells to oestradiol the underlying molecular defect responsible for the differences remains elusive. Our results suggest that ER gene regulation in SLE T cells is complex and that mechanisms that are classically associated with ER action may not fully explain the oestrogen sensitivity of the SLE T cell. The new frontier, for which little data currently exist, may require exploration into the epigenetic modification of gene targets that are somehow altered in SLE T cells.

17.14 Endocrine disruptors

As mentioned above, individuals with SLE susceptibility genes may not develop disease in the absence of environmental triggers. The release of industrial wastes into the environment has raised concerns about the potential oestrogenic-like activity of some of these synthetic chemicals [98–100]. Although several studies suggest effects of these chemicals on development and tissue-specific responses the mechanisms of action are not well understood. Xenobiotics, and specifically xeno-oestrogens, may disrupt normal endocrine function because they can bind to ERs and either inhibit or enhance ER action [101,102]. Treatment of female (NZB/NZW) F_1 mice with chlordecone accelerates the rate of SLE development [103]. Subsequent analysis of these mice indicates that chlordecone reduces total B cell and germinal centre B-cell apoptosis without affecting B cell proliferation [104].

Certain drugs have been associated with the development of a lupus-like syndrome [105] and a recent report suggests that a combination product of synthetic conjugated oestrogens can induce a similar syndrome in a post-menopausal female [106]. The study from Lascombe *et al.* [101] shows that certain xenobiotics tested *in vitro* are not able to interact with steroid receptor co-activator-1 (SRC-1). However, the test agents were able to drive ER-mediated transcription of a luciferase reporter gene transfected into MCF-7 cells, and these chemicals displaced oestradiol from the ligand-binding site, suggesting that the xeno-oestrogens were functional. A direct connection between the onset of autoimmunity and exposure to xeno-oestrogens has been difficult to evaluate because the concentration of offending agents and the time of exposure required to exert effects are difficult to establish. Nevertheless, this is an area of research that requires additional investigation and the ability to assess the genome at millions of sites may revolutionize our understanding about the relationship between environmental agents and human health [107].

17.15 Perspectives and future directions

In this chapter we have reviewed some of the evidence that the ERs will play a key role in regulating epigenetic changes in target cells. At the present time, there are few data to support the concept that altered epigenetic changes underlie the development or progression of autoimmune diseases. However, the broad scope of signalling pathways that are defective in autoimmune diseases such as SLE is consistent with some sort of a global mechanism. We have identified calcineurin [18] and CD154 [19] as oestrogen-responsive genes in SLE but not in normal T cells. Monthly administration of the ER antagonist, Faslodex (chemical name fulvestrant), over a 1-year period significantly decreased calcineurin and CD154 expression [17]. Importantly, disease activity improved in the Faslodex arm of the study and the amount of prednisone patients were taking was reduced (Table 17.2 [17]). The results are exciting but also

Table 17.2 The ER antagonist Faslodex (chemical name fulvestrant) lowered the amount of prednisone patients were taking and reduced the Systemic Lupus Disease Activity Index (SLEDAI) in female SLE patients enrolled in a 1-year study. For study details see [17].

	Faslodex arm ($n = 8$)			Placebo arm ($n = 8$)		
	Pre-study	Visit 12	Visit 15	Pre-study	Visit 12	Visit 15
Prednisone (mg/day)	5.75	2.5	2.5	5.25	10.25	10
SLEDAI	8.25	3.75	3.5	7.8	7.0	6.6

preliminary owing to the small number of patients enrolled. It is our view that lupus patients need specific therapeutic approaches that are targeted towards basic hormonal-immune deregulation and not global suppression of their immune systems. To begin to address this need we have initiated microarray profiling to identify signalling pathways in SLE patients that are abnormally regulated by oestrogen and contribute to disease activity. Blockade of these pathways with Faslodex is expected to reveal those genes and pathways, which are the most important contributors to disease activity. It is clear that alteration in the balance of co-activators and co-repressors alters the transcriptional regulation of oestrogen-responsive genes. This is an area of research that requires greater effort, particularly regarding hormone effects in the immune system. Altered co-factor recruitment may lead to abnormal epigenetic marks and may explain the abnormal oestrogenic response in SLE compared with normal T cells.

We have briefly mentioned the possibility that oestrogen mimics could be environmental agents that act as 'triggers' for disease onset. Although these chemicals are capable of binding to the ER, it is likely that the action of the ER at gene-regulatory regions is different when xeno-oestrogens are bound. Experiments to test the role of xeno-oestrogens as triggers for autoimmunity will be complicated to design. New technology including array-based approaches across the entire genome may lead to better assessment of epigenetic changes over time. Recent results suggest protein modification at arginine residues of core histones correlates with cell-fate determination in the mouse embryo [108]. CARM1 methylates arginine residues in histone H3 leaving an epigenetic mark that confers overall gene activity. CARM1 is a transcriptional co-activator of nuclear receptors, but it also operates with other transcriptional regulators to alter chromatin structure and gene activation. These results open up the possibility that epigenetic instructions may occur during early development and be involved in cell lineage determination. Although the task will be daunting, it is time to explore the role of ER recruitment of co-factors and co-repressors to target genes in the immune system (see Figure 17.2). It is important to ask whether the so-called histone code is different in SLE T cells compared with normal T cells. Both the ER and Foxp3 genes are epigenetically regulated. Are there differences in the histone code that could explain the deregulation of numerous genes that occurs in SLE? Ultimately, increased

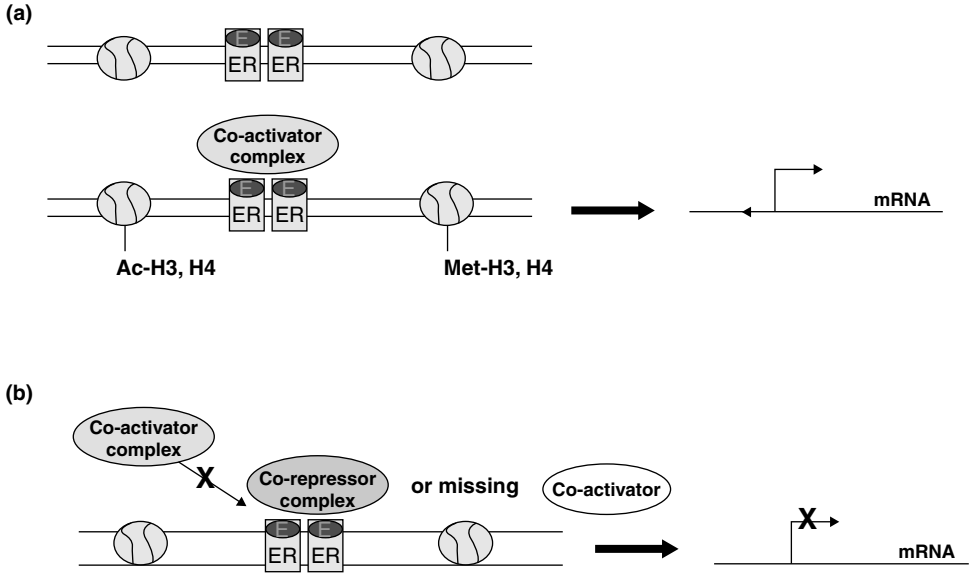

Figure 17.2 A co-activator/co-repressor epigenetic code may contribute to biased autoimmune diseases. (a) ER gene activation. Ligand-activated (E) ERs bind to target genes and stimulate the recruitment of a co-activator complex. Transcriptional activation requires the assembly of numerous co-regulatory proteins that stimulate covalent modification of histones, such as histone methylation (Met-H3, H4) and acetylation (Ac-H3, H4), and nucleosome remodelling. (b) Epigenetic repression. In autoimmune disease, deregulation could occur by the presence of a co-repressor complex that blocks the activation site or the absence of one or more co-activator components. The balance of co-factors is central to gene expression and can explain deregulation of both activation and repression mechanisms.

knowledge about epigenetic changes should provide unparalleled opportunity for new treatments because epigenetic changes could be reversible and therefore making the resulting disease treatable. The next decade of research is expected to provide new insight about the role of the ER and its participation in the development and/or progression of gender-biased autoimmunity.

17.16 Acknowledgements

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References

1. McGaha, T.L., Sorrentino, B. and Ravetch, J.V. (2005) Restoration of tolerance in lupus by targeted inhibitory receptor expression. *Science* **307**, 590–593.
2. Bouneaud, C., Kourilsky, P. and Bousso, P. (2000) Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion. *Immunity* **13**, 829–840.
3. Jacobson, D.L., Gange, S.J., Rose, N.R. and Graham, N.M. (1997) Epidemiology and estimated population burden of selected autoimmune diseases in the Untied States. *Clinical Immunology and Immunopathology* **84**, 223–243.
4. Privalsky, M.L. (2004) The role of corepressors in transcriptional regulation by nuclear hormone receptors. *Annual Review of Physiology* **66**, 315–360.
5. Khorasanizadeh, S. and Rastinejad, F. (2001) Nuclear-receptor interactions on DNA-response elements. *Trends in Biochemical Sciences* **26**, 384–390.
6. Heldring, N., Pike, A., Andersson, S. *et al.* (2007) Estrogen receptors: how do they signal and what are their targets. *Physiological Reviews* **37**, 905–931.
7. Rider, V., Jones, S.R., Evans, M. and Abdou, N.I. (2000) Molecular mechanisms involved in the estrogen-dependent regulation of calcineurin in systemic lupus erythematosus T cells. *Clinical Immunology* **95**, 124–134.
8. Rider, V., Li, X., Peterson, G. *et al.* (2006) Differential expression of estrogen receptors in women with systemic lupus erythematosus. *Journal of Rheumatology* **33**, 1093–1101.
9. Whitacre, C.C., Reingold, S.C. and O'Looney, P.A. (1999) A gender gap in autoimmunity. *Science* **283**, 1277–1278.
10. Casoli, P., Tumiati, B. and LaSala, G. (1997) Fatal exacerbation of systemic lupus erythematosus after induction of ovulation. *Journal of Rheumatology* **24**, 1639–1640.
11. Font, J., Pallares, L., Cervera, R. *et al.* (1991) Systemic lupus erythematosus in the elderly: clinical and immunological characteristics. *Annals of Rheumatic Disease* **50**, 702–705.
12. Petri, M., Kim, M.Y., Kalunian, K.C. *et al.* (2005) OC-SELENA Trial, Combined oral contraceptives in women with systemic lupus erythematosus. *New England Journal of Medicine* **353**, 2550–2558.
13. Sánchez-Guerrero, J., Uribe, A.G. and Jiménez-Santana, L. *et al.* (2005) A trial of contraceptive methods in women with systemic lupus erythematosus. *New England Journal of Medicine* **353**, 2539–2549.
14. Whitelaw, D.A. and Jessop, S.J. (2007) Major flares in women with SLE on combined oral contraception. *Clinical Rheumatology* **26**, 2163–2165.
15. Sánchez-Guerrero, J., Liang, M.H., Karlson, E.W. *et al.* (1995) Post-menopausal estrogen therapy and the risk for developing systemic lupus erythematosus. *Annals of Internal Medicine* **122**, 430–433.
16. Rider, V., Li, X. and Abdou, N.I. (2007) Hormonal influences in the expression of systemic lupus erythematosus, In: Tsokos G.C., Gordon C and Smolen J.S. (eds), *Systemic Lupus Erythematosus: a Companion to Rheumatology*, pp 87–94. Mosby, Philadelphia.
17. Abdou, N.I., Rider, V., Greenwell, C. *et al.* (2008) Fulvestrant (Faslodex) an estrogen receptor antagonist in the therapy of females with systemic lupus erythematosus: clinical, serologic, bone density, and T cell activation marker studies: a double-blind placebo-controlled trial. *Journal of Rheumatology* **35**, 797–803.
18. Rider, V., Foster, R.T., Evans, M. *et al.* (1998) Gender differences in autoimmune diseases: estrogen increases calcineurin expression in systemic lupus erythematosus. *Clinical Immunology Immunopathology* **89**, 171–180.
19. Rider, V., Jones, S., Evans, M. *et al.* (2001) Estrogen increases CD40 Ligand expression in T cells from women with systemic lupus erythematosus. *Journal of Rheumatology* **28**, 2644–2649.

20. Straub, R.H. (2007) The complex role of estrogens in inflammation. *Endocrine Reviews* **28**, 521–574.
21. Roubinian, J., Talal, N., Siiter, P.K. and Sadakian, J.A. (1979) Sex hormone modulation of autoimmunity in NZB/NZW mice. *Arthritis and Rheumatism* **22**, 1162–1169.
22. Grimaldi, C.M. (2006) Sex and systemic lupus erythematosus: the role of the sex hormones estrogen and prolactin on the regulation of autoreactive B cells. *Current Opinion in Rheumatology* **18**, 456–461.
23. Czlonkowska, A., Ciesielska, A., Gromadzka, G. *et al.* (2005) Estrogen and cytokines production-the possible cause of gender differences in neurological diseases. *Current Pharmaceutical Design* **11**, 1017–1030.
24. Gourdy, P., Araujo, L.M., Zhu, R. *et al.* (2005) Relevance of sexual dimorphism to regulatory T cells: estrogen promotes IFN-{gamma} production by invariant natural killer T cells. *Blood* **105**, 2415–2420.
25. Jenkins, J.K., Suwannaroj, S., Elbourne, K.B. *et al.* (2001) 17-β estradiol alters Jurkat lymphocyte cell cycling and induces apoptosis through suppression of Bcl-2 and cyclin A. *International Immunopharmacology* **1**, 1897–1911.
26. Miller, L. and Hunt, J.S. (1995) Sex steroids, hormone and macrophage function. *Life Sciences* **59**, 1–14.
27. Grimaldi, C.M., Hill, L., Xu, X. *et al.* (2005) Hormonal modulation of B cell development and repertoire selection. *Molecular Immunology* **42**, 811–820.
28. Abdou, N.I., Wall, H., Lindsley, H.C. *et al.* (1981) The network theory in autoimmunity: *in vitro* suppression of serum anti-DNA antibody binding to DNA by anti-idiotypic antibody in systemic lupus erythematosus. *Journal of Clinical Investigation* **67**, 1297–1304.
29. Sherer, Y., Gorstein, A., Fritzler, M.J. and Shoenfeld, Y. (2004) Autoantibody explosion in systemic lupus erythematosus: more than 100 different antibodies found in SLE patients. *Seminars in Arthritis and Rheumatism* **34**, 501–537.
30. Cutolo, M., Accardo, S., Villaggio, B. *et al.* (1993) Presence of estrogen binding sites on macrophage like synoviocytes and CD8 + CD29 + CD45RO + T lymphocytes in normal and rheumatoid synovium. *Arthritis and Rheumatism* **36**, 1087–1097.
31. Suenaga, R., Mitamura, K., Evans, M.J. and Abdou, N.I. (1996) Binding affinity and quantity of estrogen receptor in peripheral blood monocytes of patients with systemic lupus erythematosus. *Lupus* **5**, 227–231.
32. Cohen, J.H., Danel, L., Cordier, G. *et al.* (1983) Sex steroid receptors in peripheral T cells: absence of androgen receptors and restriction of estrogen receptors to OKT8 positive cells. *Journal of Immunology* **131**, 2767–2771.
33. Weusten, J.J.A.M., Blankenstein, M.A. and GmeligMeyling *et al.* 1986 Presence of oestrogen receptors in human blood mononuclear cells and thymocytes. *Acta Endocrinologica* **112** 409–414.
34. Suenaga, R., Evans, M.J., Mitamura, K. and Abdou, N.I. (1998) Peripheral blood T cells and monocytes and B cell lines derived from patients with lupus express estrogen receptor transcripts similar to those of normal cells. *Journal of Rheumatology* **25**, 1305–1312.
35. Kassi, E.N., Vlachoyiannopoulos, P.G., Moutsopoulos, H.M. *et al.* (2001) Molecular analysis of estrogen receptor alpha and beta in lupus patients. *European Journal of Clinical Investigation* **31**, 86–93.
36. Suenaga, R., Rider, V., Evans, M.J. and Abdou, N.I. (2001) *In vitro*-activated human lupus T cells express normal estrogen receptor proteins which bind to the estrogen response element. *Lupus* **10**, 116–122.
37. Kassi, E., Vlachoyiannopoulos, P.G., Kominakis, A. *et al.* (2005) Estrogen receptor alpha gene polymorphism and systemic lupus erythematosus: a possible risk? *Lupus* **14**, 391–398.

38. Johansson, M., Arlestig, L., Moller, B. *et al.* (2005) Oestrogen receptor α gene polymorphisms in systemic lupus erythematosus. *Annals of Rheumatic Disease* **64**, 1611–1617.
39. Zhao, C., Dahlman-Wright, K. and Gustafsson, J.-A. (2008) Estrogen receptor β: an overview and update. *Nuclear Receptor Signaling* **6**, 1–10.
40. Jakacka, M., Ito, M., Weiss, J. *et al.* (2001) Estrogen receptor binding to DNA is not required for its activity through the nonclassical AP1 pathway. *Journal of Biological Chemistry* **276**, 13613–13621.
41. Lahita, R.G. (2000) Sex hormones and systemic lupus erythematosus. *Rheumatic Disease Clinics of North America* **26**, 951–968.
42. Lahita, R.G., Bradlow, H.L., Kunkel, H.G. and Fishman, J. (1981) Increased 16 alpha hydroxylation of estradiol in systemic lupus erythematosus. *Journal of Clinical Endocrinology and Metabolism* **53**, 174–178.
43. Namjou, B., Kilpatrick, J. and Harley, J.B. (2007) Genetics of clinical expression in SLE. *Autoimmunity* **40**, 602–612.
44. Rhodes, B. and Vyse, T.J. (2007) General aspects of the genetics of SLE. *Autoimmunity* **40**, 550–559.
45. Simard, L. and Costenbader, K.H. (2007) What can epidemiology tell us about systemic lupus erythematosus? *International Journal of Clinical Practice* **61**, 1170–1180.
46. Deapen, D., Escalante, A., Weinrib, L. *et al.* (1992) A revised estimates of twin concordance in systemic lupus erythematosus. *Arthritis and Rheumatism* **35**, 311–318.
47. Tenbrock, K., Juang, Y.-T., Kyttaris, V.C. and Tsokos, G.C. (2007) Altered signal transduction in SLE T cells. *Rheumatology* **46**, 1525–1530.
48. Bauer, J.W., Baechler, E.C., Petri, M. *et al.* (2006) Elevated serum levels of interferon-regulated chemokines are biomarker for active human systemic lupus erythematosus. *PLoS Med*. **3**, 2274–2284.
49. Fontenot, J.D., Gavin, M.A. and Rudensky, A.Y. (2003) Foxp3 programs the development and function of CD4 + CD25- regulatory T cells. *Nature Immunology* **4**, 330–336.
50. Marson, A., Kretschmer, K. and Frampton, G.M. *et al.* (2007) Foxp3 occupancy and regulation of key target genes during T-cell stimulation. *Nature* **445**, 931–935.
51. Schubert, L.A., Jeffery, E., Zhang, Y. *et al.* (2001) Scurfin (FOXP3) acts as a repressor of transcription and regulates T cell activation. *Journal of Biological Chemistry* **276**, 37672–37679.
52. Valencia, X., Yarboro, C., Illei, G. and Lipsky, P.E. (2007) Deficient CD4 + CD25high T regulatory cell function in patients with active systemic lupus erythematosus. *Journal of Immunology* **178**, 2579–2588.
53. Polanczyk, M.J., Hopke, C. and Huan, J. *et al.* (2005) Enhanced FoxP3 expression and Treg cell function in pregnant and estrogen-treated mice. *Journal of Neuroimmunology* **170**, 85–92.
54. Tai, P., Wang, J., Jin, H. *et al.* (2008) Induction of regulatory T cells by physiological level estrogen. *Journal of Cell Physiology* **214**, 456–464.
55. Floess, S., Freyer, J., Siewert, C. *et al.* (2007) Epigenetic control of the foxp3 locus in regulatory T cells. *PLoS Biol*. **5**, 0169–0178.
56. Turner, B.M. (2000) Histone acetylation and an epigenetic code. *BioEssays* **22**, 836–845.
57. Jenuwein, T. and Allis, D.C. (2001) Translating the histone code. *Science* **293**, 1074–1080.
58. Leader, J.E., Wang, C., Fu, M. and Pestell, R.G. (2006) Epigenetic regulation of nuclear steroid receptors. *Biochemical Pharmacology* **72**, 1589–1596.
59. Leader, J.E., Wang, C., Popov, V.M. *et al.* (2006) Epigenetics and the estrogen receptor. *Annals of the New York Academy of Sciences* **1089**, 73–87.
60. O'Malley, B.W. (2007) Coregulators: from whence came these "master genes". *Molecular Endocrinology* **21**, 1009–1013.

61. Saha, A., Wittmeyer, J. and Cairns, B.R. (2006) Chromatin remodelling: the industrial revolution of DNA around histones. *Nature Reviews Molecular Cell Biology* **7**, 437–447.
62. Métivier, R., Reid, G. and Gannon, F. (2006) Transcription in four dimensions: nuclear receptor-directed initiation of gene expression. *European Molecular Biology Organization Reports* **7**, 161–167.
63. Rosenfeld, M.G., Lunyak, V.V. and Glass, C.K. (2006) Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcription response. *Genes and Development* **20**, 405–428.
64. Shang, Y., Hu, X., DiRenzo, J. *et al.* (2000) Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell* **103**, 843–852.
65. Carroll, J.S. and Brown, M. (2006) Estrogen receptor target gene: an evolving concept. *Molecular Endocrinology* **10**, 1707–1714.
66. Kininis, M., Chen, B.S., Diehl, A.G. *et al.* (2007) Genomic analyses of transcription factor binding, histone acetylation, and gene expression reveal mechanistically distinct classes of estrogen-regulated promoters. *Molecular and Cellular Biology* **27**, 5090–5104.
67. Carroll, J.S., Liu, S.X., Brodsky, A.S. *et al.* (2005) Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell* **122**, 33–43.
68. Carroll, J.S., Meyer, C.A., Song, J. *et al.* (2006) Genome-wide analysis of estrogen receptor binding sites. *Nature Genetics* **38**, 1289–1297.
69. Lonard, D.M. and O'Malley, B.W. (2006) The expanding cosmos of nuclear receptor coactivators. *Cell* **125**, 411–414.
70. Kishimoto, M., Fujiki, R., Takezawa, S. *et al.* (2006) Nuclear receptor mediated gene regulation through chromatin remodeling and histone modifications. *Endocrine Journal* **53**, 157–172.
71. Heinzel, T., Lavinsky, R.M., Mullen, T.M. *et al.* (1997) A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature* **387**, 43–48.
72. Nagy, L., Kao, H.Y., Chakravarti, D. *et al.* (1997) Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* **89**, 373–380.
73. Goodman, R.H. and Smolik, S. (2002) CBP/p300 in cell growth, transformation, and development. *Genes and Development* **14**, 1553–1577.
74. Baek, S.H., Ohgi, K.A., Rose, D.W. *et al.* (2002) Exchange of N-CoR corepressor and Tip60 coactivator complexes links gene expression by NF-kappaB and beta-amyloid precursor protein. *Cell* **110**, 55–67.
75. Gamble, M.J. and Kraus, W.L. (2007) Visualizing the histone code on LSD1. *Cell* **128**, 433–434.
76. Garcia-Bassets, I., Kwon, Y.-S., Telese, F. *et al.* (2007) Histone methylation-dependent mechanisms impose ligand dependency for gene activation by nuclear receptors. *Cell* **128**, 505–518.
77. Zwijsen, R.M.L., Buckel, R.S., Hijmans, E.M. *et al.* (1998) Ligand-independent recruitment of steroid receptor coactivators to the estrogen receptor by cyclin D1. *Genes and Development* **12**, 3488–3498.
78. Wang, C., Fan, S., Li, Z. *et al.* (2005) Cyclin D1 antagonizes BRCA1 repression of estrogen receptor alpha activity. *Cancer Research* **65**, 6557–6567.
79. Crispin, J.C., Kyttaris, V.C., Juang, Y.T. and Tsokos, G.C. (2008) How signaling and gene transcription aberrations dictate the systemic lupus erythematosus T cell phenotype. *Trends in Immunology* **29**, 110–115.
80. Feinberg, A.P. (2007) Phenotypic plasticity and the epigenetics of human disease. *Nature* **447**, 433–440.
81. Gosden, R.G. and Feinberg, A.P. (2007) Genetics and epigenetics-Nature's pen-and-pencil set. *New England Journal of Medicine* **356**, 731–733.

82. Johnson, A.B. and Barton, M.C. (2007) Hypoxia-induced and stress-specific changes in chromatin structure and function. *Mutation Research* **618**, 149–162.
83. Stearns, V., Zhou, Z. and Davidson, N.E. (2007) Epigenetic regulation as a new target for breast cancer therapy. *Cancer Investigation* **25**, 659–665.
84. Sharma, D., Blum, J., Yang, X. *et al.* (2005) Release of methyl CpG binding proteins and histone deacetylase 1 from the estrogen receptor α (ER) promoter upon reactivation in ER-negative human breast cancer cells. *Molecular Endocrinology* **19**, 1740–1751.
85. Richardson, B., Scheinbart, L., Strahler, J. *et al.* (1990) Evidence for impaired T cell DNA methylation in systemic lupus erythematosus and rheumatoid arthritis. *Arthritis and Rheumatism* **33**, 1665–1673.
86. Inui, A., Ogasawara, H., Naito, T. *et al.* (2007) Estrogen receptor expression by peripheral blood mononuclear cells of patients with systemic lupus erythematosus. *Clinical Rheumatology* **26**, 1675–1678.
87. Dhasarathy, A., Kajita, M. and Wade, P.A. (2007) The transcription factor snail mediates epithelial to mesenchymal transitions by repression of estrogen receptor-alpha. *Molecular Endocrinology* **21**, 2907–2918.
88. Kriegel, M.A., Li, M.O., Sanjabi, S. *et al.* (2006) Transforming growth factor-beta: recent advances on its role in immune tolerance. *Current Rheumatology Reports* **8**, 138–144.
89. Nawaz, Z. and O'Malley, B.W. (2004) Urban renewal in the nucleus: is protein turnover by proteasomes absolutely required for nuclear receptor-regulated transcription? *Molecular Endocrinology* **18**, 493–499.
90. Baker, S.P. and Grant, P.A. (2005) The proteasome: not just degrading anymore. *Cell* **123**, 361–363.
91. Zhang, H., Sun, L., Liang, J. *et al.* (2006) The catalytic subunit of the proteasome is engaged in the entire process of estrogen receptor-regulated transcription. *European Molecular Biology Organization Journal* **25**, 4223–4233.
92. Zhao, C., Matthews, J., Tujague, M. *et al.* (2007) Estrogen receptor beta2 negatively regulates the transactivation of estrogen receptor α in human breast cancer cells. *Cancer Research* **67**, 3955–3962.
93. Boonyaratanakornkit, V. and Edwards, D.P. (2007) Receptor mechanisms mediating non-genomic actions of sex steroids. *Seminars in Reproductive Medicine* **25**, 139–153.
94. Watanabe, M., Yanagisawa, J., Kitagawa, H. *et al.* (2001) A subfamily of RNA-binding DEAD-box proteins acts as an estrogen receptor alpha coactivator through the N-terminal activation domain (AF-1) with an RNA coactivator, SRA. *European Molecular Biology Organization Journal* **20**, 1342–1352.
95. Ko, L., Cardona, G.R., Henrion-Caude, A. and Chin, W.W. (2002) Identification and characterization of a tissue-specific coactivator, GT198, that interacts with the DNA-binding domains of nuclear receptors. *Molecular and Cellular Biology* **22**, 357–369.
96. Kammer, G.M., Laxminarayana, D. and Khan, I.U. (2004) Mechanisms of deficient type I protein kinase A activity in lupus T lymphocytes. *International Reviews of Immunology* **23**, 225–244.
97. Gorjestani, S., Rider, V., Kimler, B.F. *et al.* (2008) Extracellular signal regulated Kinase 1/2 signaling in SLE T cells is influenced by estrogen and disease activity. *Lupus* **17**, 548–554.
98. Rao, T. and Richardson, B. (1999) Environmentally induced autoimmune disease: potential mechanisms. *Environmental Health Perspective* **107**, 737–742.
99. Molina, V. and Shoenfeld, Y. (2005) Infection, vaccines and other environmental triggers of autoimmunity. *Autoimmunity* **38**, 235–245.
100. Inadera, H. (2006) The immune system as a target for environmental chemicals: xenoestrogens and other compounds. *Toxicology Letters* **164**, 191–206.

101. Lascombe, I., Beffa, D., Ruegg, U. *et al.* (2000) Estrogenic activity assessment of environmental chemicals using in vitro assays: identification of two new estrogen compounds. *Environmental Health Perspectives* **108**, 621–629.
102. Yurino, H., Ishikawa, S., Sato, T. *et al.* (2004) Endocrine disruptors (environmental estrogens) enhance autoantibody production by B1 cells. *Toxicological Sciences* **81**, 139–147.
103. Sobel, E.S., Wang, F., Butfiloski, E. *et al.* (2005) Comparison of chlordecone effects on autoimmunity in (NZB/NZW) F(1) and BALB/c mice. *Toxicology* **218**, 81–90.
104. Wang, F., Roberts, S.M., Butfiloski, E.J. *et al.* (2007) Acceleration of autoimmunity by organochlorine pesticides: a comparison of splenic B-cell effects of chlordecone and estradiol in (NZBxNZW) F_1 mice. *Toxicological Sciences* **99**, 141–152.
105. Yung, R.L., Johnson, K.J. and Richardson, B.C. (1995) A new concept in the pathogenesis of drug-induced lupus. *Laboratory Investigation* **73**, 746–759.
106. Carroll, D.G. and Cavanagh, L.E. (2007) Drug-induced lupus associated with synthetic conjugated estrogens. *Annals of Pharmacotherapy* **41**, 702–705.
107. Feinberg, A.P. (2008) Epigenetics at the epicenter of modern medicine. *Journal of the American Medical Association* **299**, 1345–1350.
108. Torres-Padilla, M.E. (2008) Cell identity in the preimplantation mammalian embryo: an epigenetic perspective from the mouse. *Human Reproduction* **23**, 1246–1252.

Abstract

We review the evidence that oestrogen (oestradiol 17-β), acting through its receptor proteins, contributes to biased autoimmunity. We also explore data supporting the concept that alterations in the histone code can induce epigenetic changes in target cells contributing to a variety of human diseases. Oestrogen receptors are viewed as key effectors of epigenetic changes because they recruit protein complexes that modify histones, alter chromatin structure and regulate target gene transcription. Inappropriate co-factor recruitment at the regulatory regions of oestrogen receptor-responsive genes may alter epigenetic marks and contribute to the development of autoimmune disease. Identification of epigenetic changes regulated by oestrogen receptors may lead to the development of specific treatments to improve hormone-associated pathologies in gender-biased autoimmune disease.

Key words: oestrogen receptors; oestradiol 17-β; gender-biased autoimmunity; epigenetics; SLE; T cells.

18

Epigenetics and systemic sclerosis

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18.1 Introduction

Systemic sclerosis (SSc) is a connective-tissue disease characterized by a heterogenous clinical picture where skin and internal organs may determine different clinical features [1]. SSc and other connective autoimmune diseases are significantly more frequent in women than in men [2,3]. Widespread fibrosis in the skin, lungs, gastrointestinal tract, blood vessels, heart and other organs is the pathological hallmark of SSc. Tissue fibrosis plays a major role in the chronic progressive clinical manifestations of the disease, and alterations in the peripheral vascular system are the earliest clinical symptoms of SSc [4]. Fibrosis, the culmination of the interplay between the immunological, vascular and fibrogenic events in the pathogenesis of SSc [5], is defined by the overgrowth, hardening and/or scarring of various tissues and is attributed to excess deposition of extracellular matrix components including collagen. Histological examination of lesional skin demonstrated a marked increase in the thickness of the dermis. Because skin thickening is manifest in most patients, researchers have analysed the molecular and biological functions of lesional skin fibroblasts that are derived from SSc patients [6], and the aberrant production of precursor interleukin (IL)-1α by skin SSc fibroblasts was associated with the induction of IL-6 and procollagen, which contributes to the fibrosis [7]. It is possible that epigenetic mechanisms are involved in collagen synthesis. They could operate at three levels: regulation of gene transcription, control of RNA stability and regulation of intracellular degradation of newly synthesized procollagen molecules before their secretion.

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18.2 Vascular alterations in SSc

In patients with SSc, dysfunction of the immune system, vascular injury and fibrosis may appear simultaneously or sequentially. Some organs may display predominantly or exclusively one feature, such as vascular disease in the kidneys, whereas other organs, including the skin and lungs, may be characterized by a mixture of inflammatory, vascular and fibrotic disease occurring at the same time or at different times. In SSc, clinical and pathological findings of vascular damage and endothelial cell activation strongly support the hypothesis of a unique vascular disease as an important and primary process. The initial events leading to vascular alterations are poorly understood but are thought to involve the endothelium with injury and proliferation, followed by an increase of smooth muscle cells, as well as deposition of interstitial matrix. Eventually, complete occlusion of the vascular lumen may develop. Evidence for endothelial cell injury in SSc includes increased circulating levels of factor VIII/von Willebrand factor, increased numbers of circulating platelet aggregates and increased levels of endothelin-1 [8–11]. Anti-endothelial cell antibodies have been reported, but they appear to be secondary, or epiphenomenal, rather than primary events. Activation of the immune system with the formation of perivascular infiltrates is another early feature of SSc. There is no evidence of immune complex deposition into the vessel walls. Although perivascular accumulation of mononuclear cells is seen in the skin, such infiltration is absent in the vascular injury of the kidneys and lungs. These infiltrates are dominated by T cells and are thought to contribute to the activation of fibroblasts by the release of profibrotic cytokines such as IL-4 [12]. The most common modifications associated with endothelial functional impairment is the alteration of leucocyte and lymphocyte adhesion proteins, whose expression is regulated by inflammatory cytokines. These molecules are cell-surface proteins involved in the cell-to-cell communications that regulate the movement of leucocytes and lymphocytes between body compartments. The loss of viable endothelial cells also leads to loss of protective and vasodilating cytokines. Nitric oxide and prostacyclin production are lost, and the action of vasoconstrictive mediators, such as endothelins, is unopposed. Loss of vascular prostacyclin favours platelet aggregation and *in situ* thrombosis. Thus the involvement of the microvasculature is functional at the beginning (reversible vasospasm) and structural in the advanced phase (obliterative vasculopathy), this latter phase being mainly due to neointimal proliferation of mesenchymal cells and deposition of interstitial matrix. It is likely that prolonged endothelial cell perturbation and activation induced by ischaemia and reperfusion may lead to dysfunction and irreversible loss of integrity, with cell detachment and persistent tissue injury. Endothelial cell damage with apoptosis resulting in the loss of capillaries is considered as one of the earliest changes in the pathogenesis of the SSc [13]. There are also attempts to compensate for the reduced capillary density leading to the formation of altered capillaries [14]. Nailfold capillaroscopy shows a variety of these morphological changes including enlarged capillaries, bushy capillary formations, microhaemorrhages and a variable loss of capillaries with or without avascular areas. Despite the reduced capillary

density there is, paradoxically, no sufficient angiogenic response in the skin of patients with SSc [15].

18.3 Tissue hypoxia, oxidative stress and SSc

One consequence of the ensuing decrease in vascular flow is lower oxygen tension in the microcirculation. Scleroderma skin is characterized by low oxygen tension [16] and the resulting hypoxia stimulates collagen gene transcription in fibroblasts, stimulates their proliferation and alters their response to growth factors [17,19]. One potential mechanism may be induction of autocrine transforming growth factor β (TGF-β) [17,18]. Thrombospondin-1 produced in response to hypoxia may further amplify TGF-β signalling by releasing active TGF-β from its complex with latency-associated peptide [20]. TGF-β, in turn, triggers a cascade of fibrotic consequences. In endothelial cells hypoxia induces enhanced transcription of the gene for platelet-derived growth factor (PDGF)-B mRNA [21], a powerful mitogen and chemotactic factor for surrounding fibroblasts. Hypoxia may further stimulate mitogenesis through upregulation of vascular endothelial growth factor (VEGF). Hypoxia also induces protective mechanisms that limit cell damage and fibrosis, particularly in the lungs. Many of these effects are mediated by the protective molecule haem oxygenase-1 (HO-1) [22,23], which protects against endothelial cell and fibroblasts apoptosis [24,25] and ensuing vascular damage [26].

Usually, tissue ischaemia leads to the expression of angiogenic growth factors (e.g. VEGF), which then initiate angiogenic sprouting by inducing vasodilatation, proliferation and migration of endothelial cells, and stabilization of the lumina to form new vessels [27]. VEGF is the major regulator of neovascularization and the elevation of plasma levels in SSc could stimulate angiogenesis [28]. The promotion of angiogenesis by VEGF is, at least in part, dependent on the vitronectin receptor, also called αvβ3 [29]. Some SSc patients showed high percentages of newly formed αvβ3-positive blood vessels, suggesting a potential for reparative angiogenesis [30].

An increase in oxidative stress is closely associated with endothelium aging, largely due to a progressive degeneration of the endogenous free radical scavengers over time. Chronic exposure of endothelial cells to reactive oxygen species (ROS) induces morphological changes and impairment of cell–cell adhesion [4,31], and oxidative stress increases vascular endothelial permeability, which is coupled with alterations in endothelial cell signal transduction. Excess generation of ROS has the ability, either directly or indirectly, to damage proteins, DNA and other cell biomolecules. Oxidative changes to proteins can lead to diverse functional consequences, such as inhibition of enzymic and binding activities, increased susceptibility to aggregation and proteolysis, increased or decreased uptake by cells, and altered immunogenicity [32]. In 63 patients affected by SSc, peroxidation products (diene conjugates) and antibodies against oxidized low-density lipoproteins were significantly increased [33]. The highest values of parameters of oxidative stress were found in the early stages, when the episodes of reperfusion after ischaemic episodes (Raynaud's

phenomenon) are very frequent. In the early stages of SSc, microvascular damage is higher than in later stages, when the number of microvessels is already significantly reduced.

Tissue hypoxia due to decreased tissue perfusion aggravates tissue injury. Silverstein *et al.* measured tissue oxygen pressure and found that SSc skin is hypoxic compared with the unaffected skin or that of control individuals [16]. In normal primary skin fibroblasts, hypoxic conditions induce collagen mRNA, suggesting that low oxygen pressure may contribute to the increased fibrogenic properties of SSc fibroblasts and to the pathogenesis of the disease [17].

18.4 Respiratory burst and post-translational modifications in SSc

ROS are key cell transducers of fibroblast proliferation and collagen gene expression [34,35]. Svegliati *et al.* observed that a pathway linking the signalling proteins Ha-Ras, growth factor–activated extracellular signal-regulated kinases 1 and 2 (ERKs 1 and 2) and ROS is amplified in SSc fibroblasts [35]. Furthermore, PDGF can induce ROS and Ras–ERK1/2 signalling [36], and IgGs derived from SSc serum reacts with human fibroblasts [37]. Baroni *et al.* demonstrated that SSc serum contains stimulatory IgGs autoantibodies directed towards the PDGF receptor [39]. These autoantibodies may trigger fibroblast activation through the PDGF receptor–ROS–ERK1/2 cascade and play a causal role in the pathogenesis of the disease [38].

The respiratory burst may be an important pathogenic factor involved in the modification of the hypoxic tissue in SSc. Therefore, endothelial damage and oxidative stress are tightly linked in SSc pathogenesis. Endothelial cells possess several defence mechanisms against oxidative stress, such as the inducible isoform of HO-1 [39]. Regarded as one of the most effective, HO-1 has cytoprotective and anti-apoptotic effects due to its production of carbon monoxide [40]. A diminished haem oxygenase activity is associated with cell death in response to oxidative stress [41]. Moreover, a blunted haem oxygenase activity exacerbates the inflammatory response in the arterial wall in animal models of atherosclerosis [23]. Similarly, HO-1 gene silencing increases ROS production, a phenomenon paralleled by an increased apoptotic cell death [42], and lack of homeostatic HO-1 upregulation fails to protect from oxidative damage and results in a higher rate of apoptotic cell death [42].

Remarkably, hypoxia has been shown to activate the expression of a number of genes at the posttranscriptional level. Panchenko *et al.* demonstrated that hypoxia induced HO-1 mRNA in SSc fibroblasts to a greater extent than in their normal counterparts [24]. The role of this alteration in the pathogenesis of SSc is unclear, but the authors hypothesized that the protective role of HO-1 overexpression in hypoxic SSc skin may account for prolonged survival of a subset of hypoxia adapted cells and, thus, contributes to skin fibrosis.

18.5 The epigenome and its environmental reprogramming

Genetics has been hypothesized to have a main role in SSc susceptibility. Yet, none of the genetic candidates is sufficient to induce the disease. For this reason, exogenous factors and epigenetic modifications have been claimed to have a role in determining the disease phenotype. This hypothesis is corroborated by the fact that the concordance rates differ significantly between dizygotic and monozygotic SSc twins [43].

In vertebrate cells the epigenome establishes gene-expression profiles. In contrast with the genome, which is identical in different cell types, the epigenome is dynamic and varies from cell to cell [44]. One of the major problems of genetics is the fact that, despite harbouring identical DNA sequences, monozygotic twins may have different phenotypes and in particular may develop different susceptibilities to diseases [45]. For this reason, an explanation has been searched outside classic genetics and the concept of epigenetics has been considered as a partial answer to these questions [46]. The term epigenetics was proposed in 1942 by Waddington, who tried to describe 'the causal interactions between genes and their products, which bring the phenotype into being' [47]. This primordial definition was shaped up some years later when epigenetics was used to define heritable changes in gene expression independent from DNA sequence alterations [48]. The epigenome comprises two different components: the chromatin structure, which is associated with the DNA, and a pattern of DNA methylation, which is part of the covalent structure of DNA [44]. Two key changes in chromatin are associated with epigenetic transcriptional repression: DNA methylation and histone modifications, including acetylation and methylation [49]. Abnormal DNA methylation at gene-transcription sites can result in epigenetic silencing of genes. Initially, it was found that DNA is hypomethylated in human tumours. This observation led to the identification of hypermethylated tumour-suppressor genes [50]. It has also been shown that DNA methylation takes place in a complex chromatin network and that this methylation is influenced by modifications in histone structure, in particular in neoplastic cells [51]. Few years ago, the inactivation of microRNA (miRNA) by DNA methylation was also demonstrated [52]. This evidence, highlighting epigenetic modifications and their contribution to the modifications of gene expression, has also suggested therapies directed at epigenetic modifications [53].

DNA methylation has critical roles in the control of gene activity and the architecture of the the cell nucleus. In humans, DNA methylation occurs in cytosines that precede guanines, called dinucleotide CpGs, that are not randomly distributed in the genome. Instead, CpG-rich regions, known as CpG islands, span the 5′ end of the regulatory regions of many genes. These islands are usually not methylated in normal cells. The methylation of particular subgroups of promoter CpG islands can, however, be detected in normal tissues [54]. DNA methylation is a powerful mechanism that may control tissue-specific gene expression. Similarly, gene-dosage reduction is involved in X-chromosome inactivation (XCI) in females [55]. Indeed, hypermethylation of

repetitive genomic sequences may prevent chromosomal instability and nuclear abnormalities are seen in cells that are missing the stabilizing effect of DNA methylation [56]. Not only do histones store epigenetic information, but DNA methylation is also linked to biochemical alterations of histone proteins [57], regulation of gene expression (transcriptional activation or repression) and DNA repair. Thus, DNA methylation and histone modifications are pivotal events that ensure normal cellular and tissular activities.

The X chromosome includes genes that are crucial for determining sex hormone levels and to maintain immune tolerance [58]. X-chromosome modifications, as clinically observed in patients with Turner's syndrome, are characterized by a marked susceptibility to developing autoimmune disorders [59]. Together with the histone code, DNA methylation is critical for XCI [60]. During this process of XCI, epigenetic regulation leads to transcription inactivation of one of the pair of X chromosomes [61], allowing the random silencing of the X chromosome inherited from either parent. In women, it has been hypothesized that a disturbance in the XCI process may be a mechanism through which lack of exposure to self-antigens may take place. In this view, skewed XCI could lead to the escape of X-linked self-antigens from presentation in the thymus or in peripheral sites that participate to induction of tolerance, to inadequate thymic deletion, and to the loss of T cell tolerance [62,63]. Importantly, two epigenetic modifications could contribute to disease development: abnormal methylation of DNA and chromatin modification of histones. Additionally, the plasticity of the epigenome enables it to be reprogrammed by interaction with the environment, in particular with chemical, physical and nutritional factors [64].

18.6 Epigenetics and SSc

In SSc, some studies have addressed the potential contribution of epigenetics to the disease's pathogenesis. Wang *et al.* investigated the effect of DNA methyltransferase and histone deacetylase inhibitors on fibroblasts, studying the expression of collagen and the level of epigenetic mediators in normal cells and in SSc fibroblasts after transient transfection with the *FLI1* gene (a suppressor of collagen transcription) or with an *FLI1* antisense construct [65]. The methylation status of the *FLI1* promoter was also verified in cutaneous biopsies derived from SSc and healthy controls. In SSc fibroblasts, the levels of epigenetic mediators, including DNA methyltransferase 1 (DNMT1), methyl-CpG DNA-binding protein (MBD) 1 and 2, methyl-CpG-binding protein 2 (MeCP-2) and histone deacetylases 1 and 6, were significantly increased, indicating DNA methylation and chromatin deacetylation in SSc fibroblasts. Two epigenetic inhibitors, 5-aza-2′-deoxycytidine and trichostatin A, were then added to SSc fibroblasts and three collagen expression genes (*smad7* (inhibitor of TGF), *p53* (endogenous repressor of TGF) and *FLI1*) were studied. Initially increased, *p53* was reduced; and *FLI1*, initially low, was increased, along with a significant reduction of proα1 collagen expression, while *smad 7* was not changed. When SSc

fibroblasts were transiently transfected with a tetracycline-regulated *FLI1* gene construct, a significant lowering of proα1 collagen expression was obtained. The addition of a *FLI1* antisense construct into normal fibroblasts resulted in a significant increase of proα1 collagen expression. When deacetylation was investigated in SSc fibroblasts, a significant reduction in the acetylated forms of histones H3 and H4 was detected. Increased methylation of the *FLI1* promoter was also detected in SSc fibroblasts and in SSc skin samples. In DNA samples, pronounced methylation of CpG islands in the SSc *FLI1* gene promoter was detected. These data suggest that the *FLI1* gene is the target of epigenetic repression in SSc fibroblasts and that it may have a pivotal role in the regulation of collagen gene expression. Histone deacetylation seems involved in epigenetic repression of FLI1 expression. All these events may lead downstream to augmented collagen synthesis by SSc fibroblasts, resulting in tissue fibrosis.

Investigation of the transactivating role of the human zinc-finger-containing transcription factor related to the *Drosophila* factor Krüppel and the Krox proteins, called c-Krox (hc-Krox), on human *COL1A1* gene in SSc dermal fibroblasts by Kypriotou *et al.* provided evidence for interactions between hc-Krox, Sp1 and Sp3 [66]. hc-Krox is a zinc-finger transcription factor belonging to the Krox family proteins and binds to GC-rich sequences. It is responsible for protein homo- and heterodimerization and for the inhibition of transcriptional activity of extracellular matrix-targeted genes [67]. hc-Krox exerted a stimulating effect on type I collagen protein synthesis, and enhanced the corresponding mRNA steady-state levels of COL1A1 and COL1A2 in SSc fibroblasts.

In SSc, skin thickening manifests in most patients, and aberrant expression of precursor IL-1α is thought to contribute to skin fibrosis. Recently, formation of precursor IL-1α complex was found to consist of precursor IL-1α, IL-1RII and HAX-1 inside SSc fibroblasts [7]. This complex plays a critical role in the fibrogenic phenotype of SSc fibroblasts. The nuclear localization of precursor IL-1α depends on the binding to HAX-1 and its biological activities might be elicited by the binding to both HAX-1 and IL-1RII in SSc fibroblasts.

One of the most potent histone deacetylase inhibitors is trichostatin A that prevents the removal of acetyl groups from core histones. Huber *et al.* [68] investigated the effect of trichostatin A on normal and SSc skin fibroblasts exposed to TGF-β. In SSc skin fibroblasts the synthesis of mRNA increased and levels of collagen 1a1, collagen1A2 and fibronectin, induced by IL-4, PDGF and TGF-β, were downregulated and abolished by trichostatin A added simultaneously with these profibrotic cyokines. TGF-β-dependent induction of a DNA-binding fraction of the profibrotic Smad3/4 was strongly abolished by trichostatin A addition. In the animal model of sclerotic skin induced by bleomycin, injection of trichostatin A reduced significantly dermal fibrosis compared to vehicle-treated control animals. This series of experiments may suggest that drugs acting on epigenetic regulation may be useful as an anti-fibrotic treatment in SSc [68].

In SSc, a disease more frequently affecting females, it has been also hypothesized that skewed XCI might have a role in its pathogenesis [69]. Indeed, the fact that

oligoclonal T cells were found in SSc skin biopsies may suggest a lack of tolerance to self antigens [70]. Ozbalkan *et al.* studied the methylation status of a highly polymorphic CAG repeat in the androgen receptor (*AR*) gene in 70 women with SSc [71]. Skewed XCI was detected in 64% of the patients and in only 8% of the control population. Specifically, extremely skewed XCI, usually a rare event defined as 90% inactivation of one allele, was present in 49% of the patients compared with only 2.4% of the controls. In skin biopsies, however, XCI was found to be random. This work demonstrates that XCI mosaicism is skewed in blood cells, but not in the skin of SSc patients. In another report, Invernizzi *et al.* studied the frequency of X chromosome monosomy in peripheral white blood cells of 44 female SSc patients [72]. They found that the frequency of X-chromosome monosomy was significantly higher in white blood cells of SSc ($6.2 \pm 0.3\%$) than in those of normal women ($2.9 \pm 0.2\%$). These data suggested that in SSc and in other polygenic autoimmune diseases multiple susceptibility genes might generate abnormal phenotypes together with epigenetic or environmental factors. The fact that autoimmune diseases may overlap in the same patient or may be present within the same family strongly suggests that genetic factors may predispose individuals to immune tolerance loss. More recently, Uz *et al.* confirmed observations on X skewing in 195 female patients with SSc [73]. They found skewed XCI in 44.9% of the patients compared with 8% of the controls. Moreover, extremely skewed X inactivation was detectable in 29.5% of the patients compared with 2.4% of controls. The authors concluded that skewed XCI mosaicism may be involved in SSc pathogenesis and that it might represent a significant risk factor for the disease [73]. However, it is clear that skewed XCI mosaicism is not sufficient to favour the onset of SSc. Clearly, other factors – genomic, viral or chemical – may overlap and contribute to the initiation of this disease [74].

18.7 Conclusions

In addition to cancer, new avenues for epigenetic cross-talk are opening in other fields, and we are far from the end of the story. Exciting recent data point to a role of epigenetic programming in behaviour and adaptation to different behavioural interactions [75]. Whole-genome approaches combining methylation and chromatin arrays have contributed to elucidating the dynamics of DNA methylation in different organs in response to the environment and in different pathologies. Understanding these processes will require knowledge of how chromatin activation leads to the reprogramming of DNA-methylation patterns. The search for additional DNA demethylases, as well as their role in cellular transformation, should become a priority in the next few years [44]. In SSc, the capacity to restore methylation is an attractive task, particularly if its effects can also be demonstrated *in vivo*. In fact, the capacity of DNA demethylases to interfere with the processes that damage the endothelium and/or induce/maintain fibrosis might become a new avenue for targeted management of SSc.

References

1. Steen, V.D. (2008) The many faces of scleroderma. *Rheumatic Disease Clinics of North America* **34**, 1–15.
2. Kast, R.E. (1977) Predominance of autoimmune and rheumatic diseases in females. *Journal of Rheumatology* **4**, 288–292.
3. Whitacre, C.C. (2001) Sex differences in autoimmune disease. *Nature Immunology* **2**, 777–780.
4. Cutolo, M., Grassi, W. and Matucci Cerinic, M. (2003) Raynaud's phenomenon and the role of capillaroscopy. *Arthritis & Rheumatism* **48**, 3023–3030.
5. Fleischmajer, R. (1995) Localized and systemic scleroderma. In: Lapière C.M. and Krieg T. (eds), *Connective Tissue Diseases of the Skin*, pp. 295–314. Marcel Dekker, New York.
6. Medsger, Jr, T.A. (2001) Systemic sclerosis (sclero derma): clinical aspects. In: Koopman W.J. (ed), *Arthritis and Allied Conditions*, pp. 1590–1624 Lippincott Williams & Wilkins, Philadelphia.
7. Kawaguchi, Y., Nishimagi, E., Tochimoto, A. *et al.* (2006) Intracellular IL-1alpha-binding proteins contribute to biological functions of endogenous IL-1alpha in systemic sclerosis fibroblasts. *Proceedings of the National Academy of Sciences USA* **26**, 14501–14506.
8. Kahaleh, M.B., Osborn, I. and LeRoy, E.C. (1981) Increased factor VIII/von Willebrand factor antigen and von Willebrand factor activity in scleroderma and in Raynaud's phenomenon. *Annals of Internal Medicine* **94**, 482–484.
9. Kahaleh, M.B., Osborn, I. and Leroy, E.C. (1982) Elevated levels of circulating platelet aggregates and beta-thromboglobulin in scleroderma. *Annals of Internal Medicine* **96**, 610–613.
10. Yamane, K., Kashiwagi, H., Suzuki, N. *et al.* (1991) Elevated plasma levels of endothelin-1 in systemic sclerosis. *Arthritis & Rheumatism* **34**, 243–244.
11. Kahaleh, M.B. (1991) Endothelin, an endothelial-dependent vasoconstrictor in scleroderma. Enhanced production and profibrotic action. *Arthritis & Rheumatism* **34**, 978–983.
12. Chizzolini, C., Parel, Y., De Luca, C. *et al.* (2003) Systemic sclerosis Th2 cells inhibit collagen production by dermal fibroblasts via membrane-associated tumor necrosis factor alpha. *Arthritis & Rheumatism* **48**, 2593–2604.
13. Sgonc, R., Gruschwitz, M.S., Dietrich, H. *et al.* (1996) Endothelial cell apoptosis is a primary pathogenetic event underlying skin lesions in avian and human scleroderma. *Journal of Clinical Investigation* **98**, 785–792.
14. Cutolo, M., Pizzorni, C. and Sulli, A. (2004) Nailfold video-capillaroscopy in systemic sclerosis. *Zeitschrift für Rheumatologie* **63**, 457–462.
15. LeRoy, E.C. (1996) Systemic sclerosis: a vascular perspective. *Rheumatic Disease Clinics of North America* **22**, 675–694.
16. Silverstein, J.L., Steen, V.D., Medsger, Jr, T.A. and Falanga, V. (1988) Cutaneous hypoxia in patients with systemic sclerosis (scleroderma). *Archives of Dermatology* **124**, 1379–1382.
17. Falanga, V., Martin, T.A., Takagi, H. *et al.* (1993) Low oxygen tension increases mRNA levels of alpha 1 (I) procollagen in human dermal fibroblasts. *Journal of Cell Physiology* **157**, 408–412.
18. Falanga, V., Qian, S.W., Danielpour, D. *et al.* (1991) Hypoxia upregulates the synthesis of TGF-beta 1 by human dermal fibroblasts. *Journal of Investigative Dermatology* **97**, 634–637.
19. Helfman, T. and Falanga, V. (1993) Gene expression in low oxygen tension. *American Journal of the Medical Sciences* **306**, 37–41.
20. Crawford, S.E., Stellmach, V., Murphy-Ullrich, J.E. *et al.* (1998) Thrombospondin-1 is a major activator of TGF-beta1 in vivo. *Cell* **93**, 1159–1170.
21. Kourembanas, S. and Faller, D.V. (1989) Platelet-derived growth factor production by human umbilical vein endothelial cells is regulated by basic fibroblast growth factor. *Journal of Biological Chemistry* **264**, 4456–4459.
22. Lee, P.J., Jiang, B.H., Chin, B.Y. *et al.* (1997) Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. *Journal of Biological Chemistry* **272**, 5375–5381.
23. Morse, D. and Choi, A.M. (2002) Heme oxygenase-1: the "emerging molecule" has arrived. *American Journal of Respiratory Cell Molecular Biologyl* **27**, 8–16.

24. Panchenko, M.V., Farber, H.W. and Korn, J.H. (2000) Induction of heme oxygenase-1 by hypoxia and free radicals in human dermal fibroblasts. *American Journal of Physiology Cell Physiology* **278**, C92–C101.
25. Petrache, I., Otterbein, L.E., Alam, J. *et al.* (2000) Heme oxygenase-1 inhibits TNF-alpha-induced apoptosis in cultured fibroblasts. *American Journal of Physiology Lung Cellular and Molecular Physiology* **278**, L312–L319.
26. Brouard, S., Otterbein, L.E., Anrather, J. *et al.* (2000) Carbon monoxide generated by heme oxygenase 1 suppresses endothelial cell apoptosis. *Journal of Experimental Medicine* **192**, 1015–1026.
27. Distler, J., Hirth, A., Kurowska-Stolarska, M. *et al.* (2003) Angiogenic and angiostatic factors in the molecular control of angiogenesis. *Quarterly Journal of Nuclear Medicine* **47**, 149–161.
28. Distler, O., Del Rosso, A., Giacomelli, R. *et al.* (2002) Angiogenic and angiostatic factors in systemic sclerosis: increased levels of vascular endothelial growth factor are a feature of the earliest disease stages and are associated with the absence of fingertip ulcers. *Arthritis Research* **4**, R11.
29. Soldi, R., Mitola, S., Strasly, M. *et al.* (1999) Role of alphavbeta3 integrin in the activation of vascular endothelial growth factor receptor-2. *EMBO Journal* **18**, 882–892.
30. Konttinen, Y.T., Mackiewicz, Z., Ruuttila, P. *et al.* (2003) Vascular damage and lack of angiogenesis in systemic sclerosis skin. *Clinical Rheumatology* **22**, 196–202.
31. Lum, H., Hao, Z., Gayle, D. *et al.* (2002) Vascular endothelial cell express isoforms of protein kinase A inhibitor. *Americal Journal of Physiology Cell Physiology* **282**, C59–C66.
32. Shacter, E. (2000) Quantification and significance of protein oxidation in biological samples. *Drug Metabolism Reviews* **32**, 307–326.
33. Simonini, G., Matucci Cerinic, M., Generini, S. *et al.* (1999) Oxidative stress in systemic sclerosis. *Molecular and Cellular Biochemistry* **196**, 85–91.
34. Sambo, P., Baroni, S.S., Padroncini, P. *et al.* (2001) Oxidative stress in scleroderma: maintenance of scleroderma fibroblast phenotype by the constitutive upregulation of free radical generation through the NADPH oxidase complex pathway. *Arthritis & Rheumatism* **44**, 2653–2664.
35. Svegliati, S., Cancello, R., Sambo, P. *et al.* (2005) Platelet-derived growth factor and reactive oxygen species (ROS) regulate Ras protein levels in primary human fibroblasts via ERK1/2: amplification of ROS and Ras in systemic sclerosis fibroblasts. *Journal of Biological Chemistry* **280**, 36474–36482.
36. Sundaresan, M., Yu, Z.X., Ferrans, V.J. *et al.* (1995) Requirement for generation of H2O2 for platelet-derived growth factor signal transduction. *Science* **270**, 296–299.
37. Chizzolini, C., Raschi, E., Rezzonico, R. *et al.* (2002) Autoantibodies to fibroblasts induce a proadhesive and proinflammatory fibroblast phenotype in patients with systemic sclerosis. *Arthritis & Rheumatism* **46**, 1602–1613.
38. Baroni, S.S., Santillo, M., Bevilacqua, F. *et al.* (2006) Stimulatory autoantibodies to the PDGF receptor in systemic sclerosis. *New England Journal of Medicine* **354**, 2667–2676.
39. Maines, M.D. (1997) The heme oxygenase system: a regulator of second messenger gases. *Annual Review of Pharmacology and Toxicology* **37**, 517–554.
40. Siow, R.C., Sato, H. and Mann, G.E. (1999) Heme oxygenase-carbon monoxide signalling pathway in atherosclerosis: anti-atherogenic actions of bilirubin and carbon monoxide? *Cardiovascular Research* **4**, 385–394.
41. Malaguarnera, L., Quan, S., Pilastro, M.R. *et al.* (2003) Diminished heme oxygenase potentiates cell death: pyrrolidinedithiocarbamate mediates oxidative stress. *Experimental Biology and Medicine* **228**, 459–465.
42. Iori, E., Pagnin, E., Gallo, A. *et al.* (2008) Heme oxygenase-1 is an important modulator in limiting glucose-induced apoptosis in human umbilical vein endothelial cells. *Life Science* **13**, 383–392.
43. Feghali Bostwick, C., Medsger, T.A. and Wright, T.M. (2003) Analysis of systemic sclerosis in twins reveal slow concordance for disease and high concordance for the presence of antinuclear antibodies. *Arthritis & Rheumatism* **48**, 1956–1963.

44. D'Alessio, A.C. and Szyf, M. (2006) Epigenetic tête-à-tête: the bilateral relationship between chromatin modifications and DNA methylation. *Biochemistry and Cell Biology* **84**, 463–476.
45. Fraga, M.F., Ballestar, E., Paz, M.F. *et al.* (2005) Epigenetic differences arise during the lifetime of, monozygotic twins. *Proceedings of the National Academy of Sciences USA* **102**, 10604–10609.
46. Sasaki, H. and Matsui, Y. (2008) Epigenetic events in mammalian germ-cell development: reprogramming and beyond. *Nature Genetics* **9**, 129–140.
47. Lujambio, A., Ropero, S., Ballestar, E. *et al.* (2007) Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. *Cancer Research* **67**, 1424–1429.
48. Holliday, R. (1987) The inheritance of epigenetic defects. *Science* **238**, 163–170.
49. Brown, R. and Strathdee, G. (2002) Epigenomic and epigenetic therapy of cancer. *Trends in Molecular Medicine* **8**, 43–48.
50. Merlo, A., Herman, J.G., Mao, L. *et al.* (1995) 5′ CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nature Medicine* **1**, 686–692.
51. Bernstein, B.E., Meissner, A. and Lander, E.S. (2007) The mammalian epigenome. *Cell* **128**, 669–681.
52. Lujambio, A., Ropero, S., Ballestar, E. *et al.* (2007) Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. *Cancer Research* **67**, 1424–1429.
53. Mack, G.S. (2006) Epigenetic cancer therapy makes headway. *Journal of the National Cancer Institute* **98**, 1443–1444.
54. Weber, M., Hellmann, I., Stadler, M.B. *et al.* (2007) Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nature Genetics* **39**, 457–466.
55. Reik, W. and Lewis, A. (2005) Co-evolution of X-chromosome inactivation and imprinting in mammals. *Nature Reviews Genetics* **6**, 403–410.
56. Xu, G.L., Bestor, T.H., Bourc'his, D. *et al.* (1999) Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. *Nature* **402**, 187–191.
57. Esteller, M. and Almouzni, G. (2005) How epigenetics integrates nuclear functions: workshop on epigenetics and chromatin: transcriptional regulation and beyond. *EMBO Reports* **6**, 624–628.
58. Gartler, S.M. and Goldman, M.A. (2001) Biology of the X chromosome. *Current Opinion in Pediatrics* **13**, 340–345.
59. Ranke, M.B. and Saenger, P. (2001) Turner's syndrome. *Lancet* **358**, 309–314.
60. Migeon, D.R., Axelman, J. and Jeppensen, P. (2005) Differential X reactivation in human placental cells: implications for reversal of X inactivation. *American Journal of Human Genetics* **77**, 355–364.
61. Lyon, M.F. (1962) Sex chromatin and gene action in the mammalian X-chromosome. *American Journal of Human Genetics* **14**, 135–145.
62. Chitnis, S., Monteiro, J., Glass, D. *et al.* (2000) The role of X-chromosome inactivation in female predisposition to autoimmunity. *Arthritis Research* **2**, 399–406.
63. Selmi, C., Invernizzi, P. and Gershwin, M.E. (2008) The x chromosome and systemic sclerosis. *Current Opinion in Rheumatology* **18**, 601–605.
64. Dolinoy, D.C. and Jirtle, R.L. (2008) Environmental epigenomics in human health and disease. *Environmental and Molecular Mutagenesis* **49**, 4–8.
65. Wang, Y., Fan, P.S. and Kahaleh, B.M. (2006) Association between enhanced type I collagen expression and epigenetic repression of the FLI1 gene in scleroderma fibroblasts. *Arthritis & Rheumatism* **54**, 2271–2279.
66. Kypriotou, M., Beauchef, G., Chadjichristos, C. *et al.* (2007) Human collagen Krox up-regulates type I collagen expression in normal and scleroderma fibroblasts through interaction with Sp1 and Sp3 transcription factors. *Journal of Biological Chemistry* **282**, 32000–32014.
67. Widom, R.L., Culic, I., Lee, J.Y. and Korn, J.H. (1997) Cloning and characterization of hcKrox, a transcriptional regulator of extracellular matrix gene expression. *Gene* **198**, 407–420.

68. Huber, L.C., Distler, J.H., Moritz, F. *et al.* (2007) Trichostatin A prevents the accumulation of extracellular matrix in a mouse model of bleomycin-induced skin fibrosis. *Arthritis & Rheumatism* **56**, 2755–2764.
69. Invernizzi, P., Pasini, S., Selmi, C. *et al.* (2008) Skewing of X chromosome inactivation in autoimmunity. *Autoimmunity* **41**, 272–277.
70. Sakkas, L.I., Xu, B., Artlett, C.M. *et al.* (2002) Oligoclonal T cell expansion in the skin of patients with systemic sclerosis. *Journal of Immunology* **168**, 3649–3659.
71. Ozbalkan, Z., Bagıslar, S., Kiraz, S. *et al.* (2005) Skewed X chromosome inactivation in blood cells of women with scleroderma. *Arthritis & Rheumatism* **52**, 1564–1570.
72. Invernizzi, P., Miozzo, M., Selmi, C. *et al.* (2005) X chromosome monosomy: a common mechanism for autoimmune diseases. *Journal of Immunology* **175**, 575–578.
73. Uz, E., Loubiere, L.S., Gadi, V.K. *et al.* (2008) Skewed X-chromosome inactivation in scleroderma. *Clinical Reviews in Allergy and Immunology* **34**, 352–355.
74. Tarr, F.K. (2008) Systemic sclerosis: the susceptible host (genetics and environment). *Rheumatic Disease Clinics of North America* **29**, 211–237.
75. Meaney, M.J. and Szyf, M. (2005) Environmental programming of stress responses through DNA methylation: life at the interface between a dynamic environment and a fixed genome. *Dialogues in Clinical Neuroscience* **7**, 103–123.

Abstract

Systemic sclerosis (SSc) is a connective-tissue disease more frequent in women than in men. Genetics have been hypothesized to have a main role in disease susceptibility, but none of the genetic factors put forward is sufficient to induce the disease. Recently, exogenous factors and epigenetic modifications have been proposed to have a role in the determination of the disease phenotype. For example, fibrotic signal transduction pathways in SSc converge on DNA methylation and histone deacetylation at the *FLI1* gene. In addition, regulation of the *COL1A1* gene in fibroblasts involves transcription factors (hc-Krox, Sp1, Sp3) which upregulate COL1A1 transcriptional activity and provide evidence for a profibrotic role of hc-Krox. These findings provide novel insights into the epigenetic regulation of fibrosis. They may suggest that the drugs acting on epigenetic mechanisms may be useful as antifibrotic treatments in SSc.

Key words: systemic sclerosis; epigenetics; vascular damage; fibroblast; genetics; X-chromosome inactivation.

19

Epigenetic regulation of B lymphocyte development and repertoire selection: relevance to autoimmunity

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19.1 Introduction

During the last few years, several developments have brought attention to a differentiated and intricate B lymphocyte contribution to the control of many aspects of the immune response. Additionally, the crucial contribution of B lymphocytes to the pathogenesis of systemic autoimmune diseases has moved into the fore. One realization is that antibodies can mediate tissue injury by a wide variety of distinct mechanisms. As the sole source of immunoglobulin, B lymphocytes, or B cells, are an essential component of the adaptive immune system, and the cellular and molecular mechanisms that control their development have been under intense scrutiny for the past two decades. In addition to their capacity to secrete autoantibodies, B cells are able to take part in various functions, such as presentation of antigenic peptides by major histocompatibility complex class II molecules to T cells [1–3]. Despite these major advances, the mechanisms that account for disruption of B cell tolerance in systemic autoimmunity remain under scrutiny. Of late, studies in several species, including plants, fruit flies, honeybees and rodents, and different model systems have revealed that diverse biological properties can be affected by epigenetic factors. In humans, epigenetic studies are pursued in a variety of conditions, including autoimmune diseases. This chapter will focus on potential epigenetic alterations that may underlie a break of tolerance in B lymphocytes, the central players in this group of diseases.

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19.2 Initiation of B cell fate choice

B lymphocytes develop from lymphoid-primed multipotent progenitors in the bone marrow that also give rise to myeloid progeny, such as macrophages and granulocytes. Molecular components must therefore regulate B cell fate 'choice' at the expense of myeloid cell fates [4]. Among these factors, the transcription factors PU.1 and CCAAT/enhancer-binding protein (C/EBP) α were demonstrated to be essential for specification of and commitment to the myeloid fate. Reversibly, the transcription factors E2A, EBF and Pax5 represent chief determinants of B cell fate, and some of them can repress expression of several genes. For the latter transcription factor, global transcriptional profiling was successfully employed to identify Pax5-repressed genes [5]. Using this approach, over 100 Pax5-repressed genes that are involved in many biological processes have been identified and validated, with the majority of these target genes normally expressed in non-B cell lineages.

In contrast to Pax5, the transcription factors E2A and EBF act in a different fashion. EBF, also called EBF-1 or Olf-1, is an atypical helix-loop-helix zinc-finger protein expressed exclusively in B lineage cells of the haematopoietic system. Targeted inactivation of E2A or EBF-1 leads to blockade of B cell development at the stage of onset of the expression of early B lineage genes and DNA rearrangements at the immunoglobulin heavy-chain (IgH) locus, suggesting that E2A and EBF function synergistically to activate the transcription of several early B lineage genes. Consistently, EBF was found to promote B cell fate by antagonizing myeloid developmental options [6]. Specification of the B cell fate also involves activation of the early B lineage genes Cd79α, Cd79β, Igll1 (λ5) and Vpreb1, which encode components of the pre-B cell receptor, and variable-to-diversity and joining (V_H-DJ_H) DNA rearrangements at the IgH locus.

19.3 Checkpoints of B cell tolerance to self

Paradoxically, in B lymphocyte development, the same B cell receptor (BcR) can either signal immunogenically, stimulating the proliferation and differentiation of B cells specific for foreign antigens, or signal tolerogenically to eliminate or silence cells that bind to self-antigens (Figures 19.1 and 19.2). Following BcR engagement, recruitment and activation of Syk is a key event in the assembly of the BcR signalosome, a complex that includes the adaptor protein BLNK (B cell linker protein) and downstream signalling components phospholipase C-γ2, Bruton's tyrosine kinase (Btk) and Rho family GTP-GDP exchange factor Vav [7,8]. Coordinately, these components induce Ca^{2+} influx and activate nuclear transcription factors, including nuclear factor of activated T cells (NFAT), activator protein 1 (AP-1) and nuclear factor κB (NF-κB), that are essential for B cell development and activation. Additionally, Casitas B lineage lymphoma (Cbl) proteins are involved in BcR signalling. This group of proteins were identified as E3 ubiquitin ligase [9], and interact with E2-ubiquitin-conjugating enzyme (Ubc) through their RING finger domain. They regulate the signalling of a broad range of receptors by

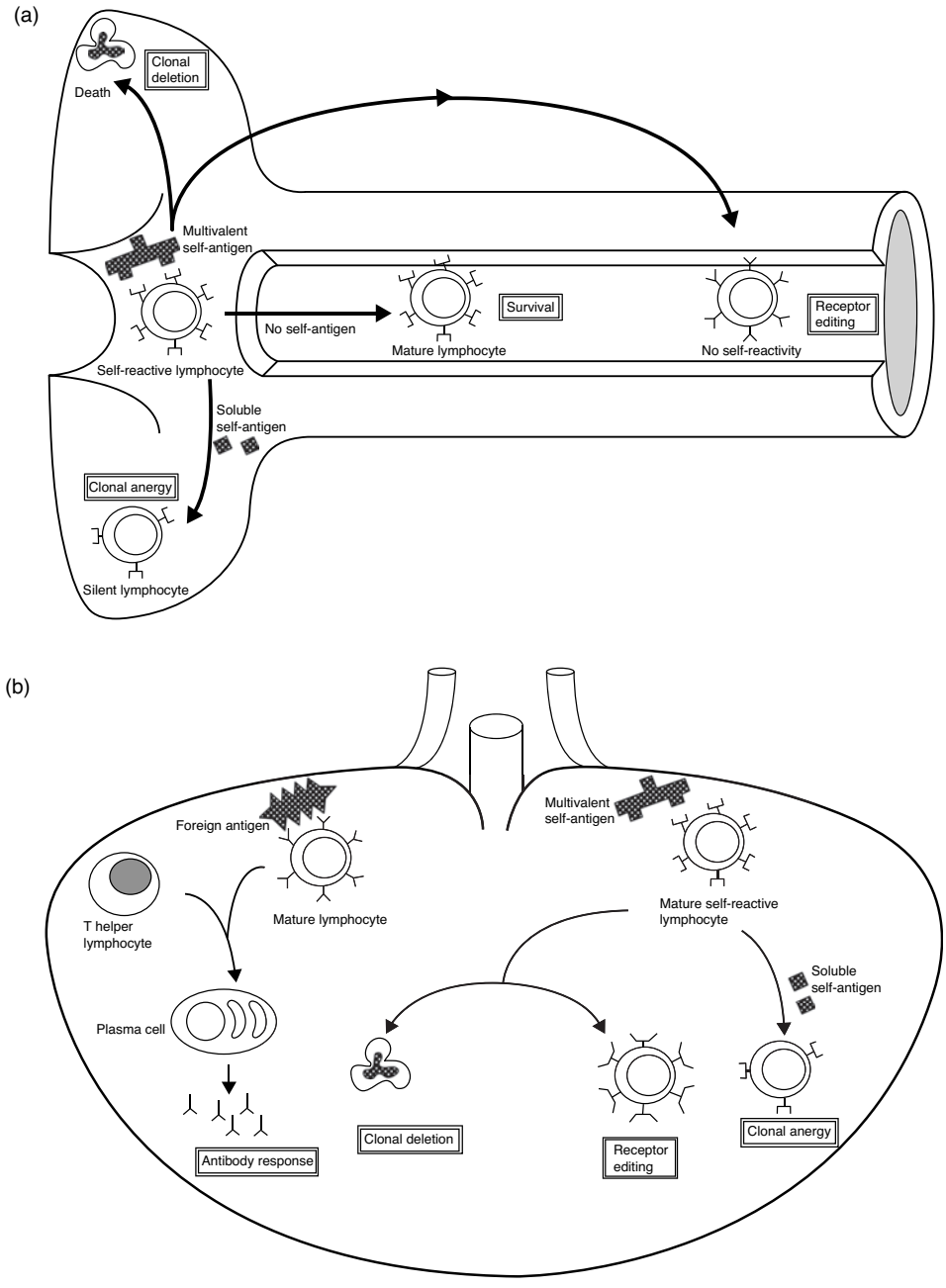

Figure 19.1 Mechanisms of B cell tolerance in the bone marrow (a) and in the periphery (b). Throughout development, the immune system can generate self-reactive B lymphocytes that must be either physically eliminated by clonal deletion, or silenced by clonal anergy or receptor editing. The nature of the self-antigen and the affinity of its interaction with the B cell receptor determine the use of a particular tolerance mechanism.

promoting ubiquitination of components involved in signalling [10]. In B cells, Cbl and Cbl-b were shown to regulate phospholipase C-γ2 activation and Ca^{2+} response. To further understand the biochemical and physiological functions of Cbl proteins in B cells, a mouse model was recently generated in which Cbl and Cbl-b were simultaneously inactivated in B lineage cells [11]. The mutations substantially increased the rate of B cell maturation and impaired B cell anergy, and the mice manifested systemic lupus erythematosus (SLE)-like disease, suggesting that Cbl proteins control a checkpoint of B cell tolerance, possibly by extending the duration of B cell maturation, and providing sufficient time for the induction of B cell tolerance [11].

19.4 Negative regulation of immunoglobulin gene joining

Since immunoglobulin gene assembly is a central event in the life of B cells, understanding the mechanisms that maintain immune tolerance in the B cell compartment requires a detailed understanding of immunoglobulin gene rearrangement and its regulation. In B lymphocyte biology, the mouse Igκ locus represents a suitable system for studying the relationship between chromatin structure and gene regulation [12]. Spanning more than 3.2 Mb, this locus is the largest multigene family thus far identified with respect to genomic length [13]. It contains 95 potentially functional V_κ genes that have been categorized into 18 families based on sequence homologies, four functional and one non-functional J_κ regions, and a single C_κ exon. Genetic elements that regulate rearrangement of the κ locus include enhancers in the intron (iEκ) and 3′ of the κ constant region (3′Eκ). The probability that the κ locus will recombine also correlates with the appearance of so-called germline transcripts that initiate within unrearranged V_κ genes. Numerous ubiquitous and B cell-specific transcription factors have been shown to bind the κ enhancers at some point during B cell differentiation. In particular, the E2A transcription factors, as well as the interferon regulatory factor-4 (IRF-4) and the closely related IRF-8 appear to be important for the regulation of locus accessibility and gene rearrangement [14].

Several *cis*-acting regulatory elements have been identified in the Igκ locus, including transcriptional promoters and enhancers, as well as elements that positively regulate gene rearrangement. Epigenetic differences in the chromatin structure of the 'accessible' allele have been observed, including histone modifications and alterations in subnuclear localization [15]. However, additional elements that specify various forms of negative regulation – including those responsible for conferring either B or T cell specificity for gene rearrangement, for blocking rearrangement in pro/pre-B cells during development and for governing the silencing of allelically excluded alleles – remain to be unidentified. One such candidate element that could participate in negative regulation is a pro/pre-B cell transcriptional silencer, earlier identified in the intervening sequence between the closest V_κ gene and the J_κ region and termed Sis (silencer in the intervening sequence). This *cis*-acting element has been identified in the region between the V and joining (J) gene segments of the κ locus. It associates

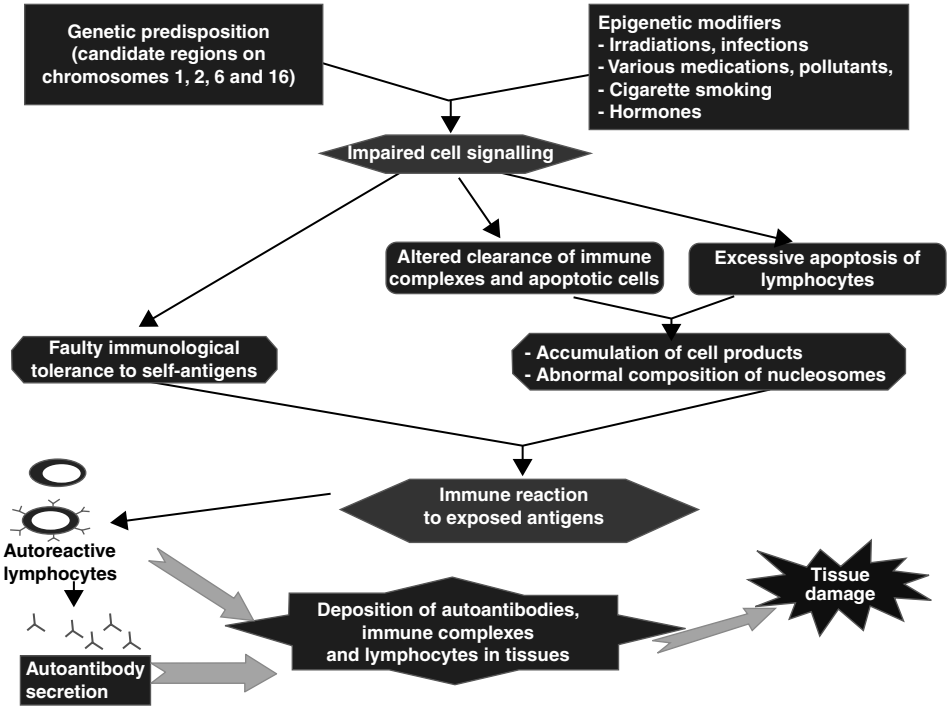

Figure 19.2 Model for the emergence of pathogenic autoreactivity in the B cell compartment. On a genetically predisposed individual, epigenetic factors can lead to subversion of the signalling pathways that normally allow lymphocytes to mount potent responses to potential threats while remaining tolerant to self. Such alterations in signalling have been shown to result in disruption of lymphocyte tolerance to self, and clinical manifestations of autoimmunity in experimental models.

with Ikaros and targets κ transgenes to centromeric heterochromatin, thus negatively regulating κ gene rearrangement. It also has regulatory functions that require its presence near the J-C region in germline alleles. That Sis negatively regulates V-J joining during B cell maturation makes it the first example of an endogenous sequence that silences immunoglobulin gene V-J joining specifically in B lymphocytes [16].

19.5 B cell fate commitment and immunoglobulin gene accessibility

B lineage precursors are generated from haematopoietic stem cells through an ordered developmental pathway controlled by a hierarchical and dynamic regulatory network that includes the cytokine receptors for Flt3 ligand and interleukin 7 (IL-7), and the transcription factors PU.1, Ikaros, Bcl11a, E2A, EBF and Pax5. Among those factors, EBF and Pax5 are obligate factors because their functions cannot be bypassed in simple

complementation assays. They serve important functions in B cell fate specification and commitment by activating B lineage genes and concomitantly repressing those of alternative lineages. EBF induces Pax5, and initiates the restriction of alternative lineage options and B cell fate commitment independently of Pax5 [17]. Together, EBF and Pax5 directly control three distinct molecular processes in early B cell development: activation of B lineage-specific genes, repression of alternative lineage genes, and rearrangement of the IgH locus.

Recombination activating genes *RAG1* and *RAG2* are tightly linked in the genome and are transcribed in concert, and distinct *cis*-acting elements and transcription factors regulate their expression in developing B lymphocytes and T lymphocytes [18]. A second level of regulation of V(D)J rearrangement involves control of accessibility of immunoglobulin or T cell receptor gene segments to the recombinase [19]. Both large-scale and localized molecular changes are believed to control the sequential and selective accessibility of various regions of the IgH locus to the recombinase. Accessibility and recombination of immunoglobulin heavy-chain variable region (V_H) gene segments have been correlated with the repositioning of the IgH locus from the nuclear lamina to the centre of the nucleus and its compaction to generate DNA loops [20,21]. Localized events regulating accessibility include changes in chromatin structure through histone modification and activation of germline as well as antisense transcription [22,23].

As in other cell types, B cell fate commitment is not simply a consequence of the action of a single transcription factor. Instead, it depends on a network of regulatory molecules. The transcription factor signal transducer and activator of transcription 5 (STAT5) and the chromatin modifier Ezh2 regulate accessibility of distal V_H gene segments through distinct non-redundant molecular mechanisms. Ikaros, on the other hand, is essential for positively regulating V_H-to-DJ_H rearrangement at the IgH locus, which is the defining hallmark of B cell fate commitment [24]. Through binding *in vitro* and *in vivo* to sites in the *RAG* locus, and regulating histone acetylation, as well as transcription, Ikaros serves a central function in immunoglobulin gene rearrangement at the pro-B stage by simultaneously regulating the expression of *RAG1* and *RAG2* genes, and controlling V_H gene accessibility and compaction of the IgH locus [24].

19.6 Changes in chromatin structure during B cell development

During early embryogenesis, tissue-specific genes are put into a repressed epigenetic state that is then maintained during development. Their activation in a cell-type-specific fashion requires 're-opening' that involves sequential removal of multiple layers of repression [20,25]. During B cell development, several epigenetic mechanisms were found to be involved in the repression of V(D)J recombination. To reach an accessibility threshold that will allow the cell to initiate immunoglobulin gene assembly, each locus must be de-repressed in a programmed way. For the κ locus, gene activation

is associated with a programmed series of epigenetic changes that take place in a stepwise manner, ultimately rendering one Igκ allele preferentially accessible to rearrangement [15]. These events proceed first at the level of nuclear organization, then at the level of chromatin (histone modification) and finally at the level of DNA (demethylation). As seen in other cell types, inactivation of many genes is associated with their physical recruitment to heterochromatin foci and nuclear organization is an important parameter in this process.

Three-dimensional fluorescence *in situ* hybridization analysis has shown that the Igκ locus undergoes dynamic changes during lymphoid development [26]. In very early lymphoid progenitor cells, both Igκ alleles seem to be closely associated with the nuclear periphery, but progression to the pro-B cell stage is associated with a repositioning into the body of the nucleus, in regions not associated with heterochromatin domains [26]. Genes that become inactivated in the B cell lineage seem to be relocated to heterochromatin regions, where they are associated with Ikaros, a protein known to mediate gene repression. Whereas Igκ sequences appear to be positioned near the periphery of the nucleus, both alleles become centrally located at the pro-B stage of development [20]. It is thought that lineage-specific association with the nuclear periphery is involved in repressing rearrangement, and relocation to the centre is likely to render the Igκ locus slightly more accessible. In addition to relocation to a repressive chromatin environment, other epigenetic factors are likely to mediate changes that affect the accessibility of a gene and alter its function, including overall chromatin conformation, as measured by DNase I sensitivity, DNA methylation and histone modification.

Using a variety of cell types representing different stages of B cell development, it was demonstrated that one of the first markers of chromatin accessibility is set up in the pro-B cell stage [15]. This change is noted when Igκ gene sequences are moved from the periphery to the centre of the nucleus. Their nuclear reorganization event is accompanied by changes in local chromatin structure, such as histone acetylation and methylation of histone H3 at Lys-4 (H3-K4). As pre-B cell development proceeds, one Igκ allele was found to be recruited to centromeric heterochromatin, in intimate association with proteins linked to gene silencing (Ikaros and heterochromatin protein 1-γ) [15]. The changes observed render the other Igκ allele susceptible to *trans*-acting factors, such as RelB, which can mediate DNA demethylation, and thus allow immunoglobulin gene recombination directly or indirectly by the recruitment of other factors.

As discussed above, Igκ genes undergo considerable epigenetic changes even though the Igκ locus itself remains fully methylated throughout pre-B cell development [15]. This observation raised the possibility that chromatin alterations in *cis* are involved in controlling accessibility of the Igκ locus to the DNA demethylation machinery. In fact, it appears that in pre-B cells the Igκ locus undergoes chromatin changes in *cis* that render one allele more susceptible to the *trans*-acting factors that assist in bringing about DNA demethylation in B cells [15]. It could be that the relocalization of Igκ to heterochromatin involves association with Ikaros [15], a protein that forms multimers able to colocalize with foci of pericentromeric heterochromatin in pre-B cells. As has

been previously suggested, Ikaros may be involved in the transcriptional inactivation of specific genes in the B lymphoid lineage [27].

In addition to nuclear reorganization, pre-B cell development is also characterized by progressive changes in histone modification, including general acetylation and the specific methylation of H3-K4 at both the Igκ and IgH loci [28,29]. This process was found to occur preferentially on the non-heterochromatin allele [15], but the mechanism for this selectivity is not known. It may be that, before recruitment, both Igκ alleles are present in an equally accessible and acetylated form, and that deacetylation/recruitment of one Igκ allele at the small pre-B cell stage directs the differential regulation of the two alleles.

19.7 Epigenetic changes through association of different immunoglobulin loci

In lymphocytes, common factors are required for V(D)J recombination at immune receptor loci, and developmentally regulated changes in locus accessibility are therefore crucial for regulating this process. Recent studies have shown that dynamic movement of the immunoglobulin loci exerts control of recombination at many levels [12]. For example, locus contraction mediated by looping occurs before the onset of Igκ germline transcription. Repositioning of one Igκ allele to pericentromeric clusters occurs at the pre-B cell stage, before the onset of Igκ rearrangement, and may limit accessibility of the Igκ locus to the recombinase to a single allele. In contrast, successful recombination of one allele in the IgH locus is followed by repositioning of the second allele to pericentromeric heterochromatin, a repressive compartment of the nucleus, resulting in a decreased accessibility of the IgH locus to the recombinase during Igκ rearrangement. In addition, 'decontraction' of the IgH locus occurs at the same developmental stage. This process contributes to allelic exclusion by physically separating distal and middle V_H gene segments from the proximal D-J domain of the IgH locus, thereby preventing further synapse formation and ongoing rearrangement between these regions.

The mechanisms underlying locus contraction are being investigated. The transcription factor Pax5, together with another unknown factor, are required at the IgH locus [30]. Remarkably, it was recently found that IgH locus decontraction depends on association with the Igκ locus at pericentromeric regions, and that pericentromeric repositioning in the absence of Igκ association does not induce decontraction [31]. This demonstrated interaction between the IgH and Igκ loci involves interchromosomal association occurring at pericentromeric heterochromatin, and is a transient event that is important for early silencing of one IgH allele in pre-B cells and for changing its conformation [31]. It will be useful to define the precise regions of the IgH locus that mediate its association with Igκ and to identify the proteins that mediate this association [31].

The pericentromeric repositioning of the IgH locus at the immature B cell stage [31] suggests that at later stages of development additional mechanisms – such as a decrease

in IL-7 and subsequent deacetylation, a modification known to decrease accessibility at the IgH locus – enforce silencing of the IgH locus [32]. These observations are reminiscent of studies showing that transcription of genes located on one chromosome can be controlled by regulatory elements located on a different chromosome. Examples include an association between the promoter of the gene encoding the T helper (Th) type 1 cytokine interferon-γ and the locus-control region of genes encoding Th2 cytokines [33].

Thus, it appears that the immunoglobulin loci are not static [31]. After they have repositioned to pericentromeric regions at the pre-B cell stage of development, they do not remain in the same nuclear location as the associated alleles do, but move apart to different pericentromeric clusters at the immature B cell stage. One possibility is that alleles shuttle between the periphery and pericentromeric heterochromatin, two environments thought to be repressive. That association between the Igκ and IgH loci is mediated by the 3′ Igκ enhancer can induce decontraction of the IgH locus in pre-B cells [31] is intriguing. It adds a further layer of complexity to immunoglobulin gene regulation and may indicate that association of the two loci can lead to an epigenetic change that alters locus conformation.

19.8 Epigenetic factors that allow full utilization of the immunoglobulin repertoire

To determine whether DNA sequences immediately surrounding V_H gene segments are sufficient for the proper regulation of V_H to DJ_H rearrangement or whether the regulation of rearrangement depends on the chromosomal position and the context of a V_H gene segment, gene targeting was used to reposition a distal V_H gene segment to a region just upstream of the V_H gene cluster [34]. Targeting a distal V_H gene segment to a region 1 kb upstream of the D_H gene *DFL16.1* caused it to recruit activating chromatin modifications, to rearrange more frequently than its endogenous counterpart, to rearrange directly to unrearranged D_H gene segments, to violate allelic exclusion and to lose lineage specificity. Thus, chromosomal position can profoundly affect the regulation of V_H gene rearrangement. These observations [34] are compatible with the view that locus contraction, mimicked by proximal targeting, can override regulation imposed by DNA sequences immediately surrounding V_H gene segments.

Changes in the structure of the chromatin surrounding V_H genes are likely to regulate the accessibility of the recombination signal sequences to undergo RAG recombinase binding and subsequent recombination [35]. Such chromatin changes can involve histone post-translational modifications and ATP-dependent nucleosomal remodelling. Because the phenotype of Ezh2-deficient mice suggested that methylation of H3-K27 may play a crucial role in controlling distal V_H gene rearrangement [36], one study compared the methylation status of distal and proximal V_H genes to determine whether they differ [37]. Chromatin immunoprecipitation (ChIP) and ChIP-on-chip experiments revealed that the repressive H3-K27me post-translational modification is observed exclusively on the proximal V_H genes. In addition, H3-K36me – a

post-translational modification associated with active genes [35] – is greatly enriched on distal V_H genes and is also present to a lesser extent in B-lineage cells on proximal V_H genes, thus displaying the reciprocal pattern of expression to H3-K27me3 [37]. Two histone post-translational modifications, H3-K27me3 and H3-K36me2, are unique in that they show equal or higher enrichment on a subset of V_H genes than on D_H or J_H genes, and most importantly reciprocal patterns of expression on distal and proximal genes. These reciprocal epigenetic patterns of H3-K36me and H3-K27me suggest that these modifications may act in concert to promote rearrangement of all V_H gene families.

Previous studies had shown that transgenic expression of Pax5 in thymocytes was sufficient to activate V_H to DJ_H rearrangement and cause compaction of the distal and proximal regions of the IgH locus [30,38]. Pax5 had also been shown to be necessary for removal of inhibitory histone methylation around the distal V_H gene segments [34], suggesting that Pax5 regulates V_H to D_H rearrangement by bringing distal V_H gene segments into proximity with D_H segments (compaction) [34]. Consistently, analysis of IgH locus recombination in Pax5-deficient pro-B cells revealed that whereas D-to-J recombination is normal, V_H gene usage exhibits a progressive 3′ to 5′ defect very similar to that reported for IL-7 receptor-deficient mice [39]. Recombination of distal V_H gene families (3609, J558) was strongly reduced. In striking contrast to previous findings in IL-7 receptor-deficient mice, however, accessibility, as measured by sterile transcription and histone acetylation of both 3′ and 5′ V_H genes, was equivalent in wild-type and *Pax5*$^{-/-}$ pro-B cells. Hence, Pax5 appears to play a role in regulating V(D)J recombination of the IgH locus at a level distinct from histone acetylation and germline transcription. Together, the cytokine IL-7 and the transcription factor Pax5 seem to be essential to set the epigenetic profile that permits full utilization of the distal and proximal V_H genes to create a diverse antibody repertoire.

19.9 Multistep regulation of B cell maturation

Differentiation of B lymphocytes to plasma cells is a remarkable example of transformation that necessitates adjustment of cell architecture to comply with new functions. Resting B lymphocytes express, but do not secrete, immunoglobulins on their surface as clonal antigen receptors. They have a small cytoplasm, with scarce endoplasmic reticulum cisternae. Upon BcR engagement, B cells proliferate and differentiate into plasma cells, each of which secretes thousands of immunoglobulins per second.

To follow the differentiation process with time and to investigate how B lymphocytes reorganize their machineries to become professional secretors, a dynamic proteomics approach was used to follow the transformation from dormant B lymphocytes to plasma cells [40]. These studies disclosed that sequential waves of proteins are coordinately expressed during the transformation of B cells into plasma cells. On the

first day of activation, mitochondrial and cytosolic chaperones show high expression, whereas metabolic enzymes peak on the third day. Endoplasmic reticulum-resident proteins increase linearly during differentiation, accompanied by proteins involved in the redox balance. A sharp increase in IgM synthesis occurs only after 2 days of activation. Thus, the transformation from dormant B cells to secretory plasma cells is a multistep process. Upon activation, B cells carefully prepare for their role as plasma cells well before IgM secretion starts. They ensure that their metabolic capacity and their secretory machinery have expanded enough to accommodate the launch of IgM production [40].

19.10 Altered B cell functions in systemic autoimmunity

SLE is considered a prototype of systemic autoimmune disease [41]. Both in human and in murine models of the disease, B cell overactivity is responsible for hypergammaglobulinaemia and the production of a large variety of autoantibodies, some of which are convincingly involved in the pathogenesis of immune complex-mediated histological damage [3]. Different factors contribute to lupus B cell overactivity, including defective regulatory T cell subsets, excessive help provided by certain T cell subsets, defective Fcγ receptor-mediated suppression and overreactivity to cytokines delivered to B cells in an endocrine, paracrine or autocrine fashion.

Despite the importance of B cells in the pathogenesis of SLE, the immune abnormalities that lead to the activation and differentiation of self-reactive B cells into autoantibody-producing cells in humans have not been identified. In normal individuals, anergic autoreactive B cells are removed from the peripheral B cell repertoire at the T1 to T2 transition stages, never advancing into the mature B cell subset [1]. In patients with lupus, however, proportions of self-reactive B cells in the mature naive B cell compartment are increased, as compared with normal controls [42,43]. Notably, B cell-activating factor belonging to the tumour necrosis factor family (BAFF) has been shown to play a critical role in this process, with high serum levels of BAFF enhancing survival of self-reactive immature B cells and promoting their entry into the mature B cell pool [44]. Thus, patients with SLE have a disturbance of tolerance resulting in an increased proportion of the naive B cell population becoming activated.

B cells from lupus patients are hyper-responsive to a variety of stimuli, demonstrating enhanced proliferation to polyclonal activators, increased anti-IgM- and anti-IgD-mediated intracellular Ca^{2+} responses, increased anti-IgM-induced protein tyrosine phosphorylation and decreased FcγRIIb1 inhibition of BcR signalling [8,45]. Lupus patients also have increased proportions of B cells expressing activation markers, with elevated expression of CD86 seen in both small 'resting' and large 'activated' B cells. The abnormal activation of B cells in lupus does not appear to reflect disease activity; nor does it arise from drug treatment effects, suggesting that it reflects an intrinsic B cell defect [46]. Although increased B cell activation was seen in

both the $CD27^-$ and $CD27^+$ B cell compartments of lupus patients, the most dramatic changes were within the 'putatively' naive $CD27^-$ B cell subset. Furthermore, the increased B cell activation in lupus is not just due to accumulation of B cell populations that are normally activated, but arises from enhanced activation of multiple B cell populations, many of which demonstrate minimal activation in healthy controls. It is possible that the increased co-stimulatory molecule expression results from a defect in the induction of anergy in the self-reactive B cells of individuals with lupus.

In fact, evidence gathered from the study of both the human disease and its murine models suggests that B cells may be intrinsically defective. For example, unmanipulated lupus B cells display aberrant BcR-induced cell signalling events, which may contribute to the abnormal function of SLE B cells [8,45]. Thus, fundamental disorders of the cellular immune response in SLE are 'intrinsic' B cell defects in cell-surface molecules and cytokine production and responsiveness [45]. For example, in contrast to normal B cells, freshly isolated SLE B cells display a higher epitope density of cell-surface CD40, CD86 and BAFF receptor and spontaneously produce increased amounts of cytokines (IL-10, IL-6).

19.11 Impaired B cell tolerance to self in systemic autoimmunity

Two important mechanisms of tolerance are defective in this group of disorders. First, studies of anergy indicate that this tolerance mechanism is deficient in systemic autoimmunity [47]. Second, patients with rheumatic autoimmune diseases, including rheumatoid arthritis and SLE, fail to efficiently remove autoreactive B cells at both early and mature tolerance checkpoints, in the bone marrow and in the periphery, and exhibit alterations of receptor editing, a chief mechanism of B cell tolerance [48–50]. It is easy to envision that this altered editing may promote disease by an uncontrolled creation of autoreactive antibodies. It is also conceivable that impaired receptor editing in SLE and rheumatoid arthritis results in ineffective silencing of B cells that have acquired autoreactive receptors. Studies of lupus susceptibility loci suggest that impairment of receptor editing may be a major mechanism through which antinuclear antibodies arise [51–54].

Converging observations indicate that receptor editing seems to contribute to the pathogenesis of rheumatic autoimmune diseases in human through at least two different mechanisms [50]. Whereas impaired secondary rearrangements may result in ineffective silencing of B cells, exacerbation of receptor editing can give rise to the emergence of autoreactive receptors from clones that were initially devoid of autoreactivity. Both alterations can promote the pathogenesis of autoimmune diseases by favouring the uncontrolled emergence and/or persistence of autoreactive B cell clones. Additionally, other mechanisms, such as clonal deletion or anergy, may be disturbed so as to contribute to autoreactivity formation.

19.12 Epigenetic factors underlying impaired B cell tolerance

Several observations point to the existence of altered transcriptional regulation in SLE lymphocytes, with increased levels and types of defective transcripts relative to normal individuals, an abnormality that could alter functions of their lymphocytes [55–57]. Thus, studies of forkhead transcription factors (FOXO) that regulate cell proliferation and activation, cell survival and B cell class-switch recombination revealed that FOXO1 transcript levels are decreased in SLE patients with active disease activity [56], suggesting that deregulation in gene expression may be connected to the development of tissue damage mediated by the over-activation of autoreactive B lymphocytes.

One mechanism that might account for the skewed expression of CD40, IL-10 and CD86 in SLE B cells is altered chromatin structure, in part as a result of an imbalance of histone acetylation and or methylation. It can be hypothesized that deficient acetylation and/or methylation exist and result in skewed gene expression in SLE B cells. It will be useful to test whether anti-IgM treatment of human B cells from SLE patients and control individuals leads to dynamic changes in histone H3 lysine acetylation and whether it affects the status of chromatin histone lysine methylation, particularly histone H3 Lys-4 dimethylation (H3K4me2) and Lys-9 dimethylation (H3K9me2). It will also be useful to determine whether altered expression of CD40, IL-10, BAFF receptor and C86 is associated with relative under-acetylation, and whether increasing histone acetylation by inhibiting histone deacetylase activity might correct the disorder. The possibility that aberrant expression of the encoding genes in SLE B cells reflects skewed gene expression because of altered acetylation of these genes could be tested by probing the capacity of histone acetylation inhibitors to reverse this autoimmune disorder.

As several studies indicate, it is conceivable that impaired receptor editing in SLE and rheumatoid arthritis could result in ineffective silencing of B cells that have acquired autoreactive receptors [48,50,58–61]. Further insight into the molecular basis of inefficient editing as a potential cause of autoimmunity comes from studies based on different approaches. First, studies of two lupus susceptibility loci suggest that impairment of receptor editing may be a major mechanism through which anti-nuclear antibodies arise in lupus [52,54]. For example, *Sle2*^{z} is a NZM2410-derived locus on mid-chromosome 4, which impacts autoantibody production and lupus nephritis. When introgressed onto the normal C57BL/6 background, the *Sle2*^{z} locus leads to age-dependent hypergammaglobulinemia and generalized B cell hyperactivity. To understand how *Sle2*^{z} breaches B cell tolerance, this locus was bred to two anti-DNA immunoglobulin H-chain transgenic models, B6.3H9 and B6.56R [54]. The introgression of the *Sle2*^{z} lupus susceptibility interval onto C57BL/6 mice carrying an anti-DNA heavy chain-chain transgene augmented the breach in B cell tolerance to DNA. Additionally, anti-nuclear antibodies exhibited diminished receptor editing in the context of the transgene. The data suggest that the presence of *Sle2*^{z} may diminish the

pressure to edit an initial BcR that may have been autoreactive, and that impairment of receptor editing may be a major mechanism through which anti-nuclear antibodies arise in lupus.

A second line of evidence comes from investigation of the effect of hydralazine, an anti-hypertensive drug that triggers lupus in humans, on receptor editing in mice harbouring human transgenic immunoglobulins. The studies revealed that, by disrupting the extracellular-signal-regulated kinase (ERK) signalling pathway, hydralazine reduces receptor editing in B lymphocytes and contributes to generation of pathogenic autoreactivity [62]. The data also support the view that epigenetic alterations contribute to exacerbated activation or deregulation of the mechanisms that maintain tolerance to self-antigens in patients with lupus, a systemic autoimmune disease that can be triggered by medications taken to treat a variety of conditions. Future studies will determine whether other drugs and xenobiotics that trigger autoimmunity in humans also can act through similar mechanisms.

19.13 Future prospects

The potential roles of molecular processes and subsequent selective influences on the generation of autoreactive B cells remain the focus of investigation. Since epigenetic mechanisms can affect various biological properties – from the eye colour of fruit flies to the morphology of flowers [63] – further investigation into the impact of epigenetics on lymphocyte tolerance to self might provide us with unexpected clues to elucidate more fully the basis of dysregulated receptor editing in autoimmune disease.

In the mouse, a DNA element predicted to have an exclusive role in receptor editing is the recombining sequence (RS), the murine homologue of the human Igκ-deleting element (IGKDE). RS lies 25 kb downstream of C_κ, carries a canonical immunoglobulin gene recombination signal, and rearranges by V(D)J recombination to V_κ elements and to sites in the J_κ-C_κ intron. RS recombination results in deletion of the C_κ exon and silencing of the Igκ locus. Almost all mouse and human λ B cells (75–95%) and 10–15% of κB cells carry RS recombinations [64]. Compared to mice, humans have a higher fraction of B cells that express a λ chain (40 compared with 6%) or rearrange IGKDE (50 compared with 20%) [64]. In actively rearranging B cell lines, RS, IGKDE and Igλ recombinations are temporally correlated, but with rearrangements of RS preceding those of Igλ [65].

Indirect arguments led to the view that RS and IGKDE play roles in key physiological processes, such as light-chain isotype exclusion and receptor editing. Recently, a mouse strain was generated in which the recombination signal of RS was removed (Δ), blocking RS-mediated Igκ inactivation [66]. The RSΔ mutation blocked RS recombination, and allowed evaluation of its putative functions. RSΔ/Δ mice had poor λ B cell production, but surprisingly normal κ λ exclusion. Most importantly, RSΔ/Δ mice had a targeted defect in receptor editing that abrogated tolerance and promoted autoantibody formation, particularly in conjunction with an apoptotic defect contributed by Bcl_2 transgenic expression.

Other observations indicate that transcription factors serve a central function in rearrangement of the immunoglobulin locus by simultaneously regulating the expression of *RAG1* and *RAG2* genes, and controlling V_H gene accessibility and compaction of the IgH locus [24]. For example, Ikaros binds *in vitro* and *in vivo* to many sites in the *RAG* locus, and regulates histone acetylation as well as transcription. Additional studies revealed that transcription of genes located on one chromosome can be controlled by regulatory elements located on a different chromosome [15], and that the cytokine IL-7 and the transcription factor Pax5 are essential to set the epigenetic profile that permits full utilization of the distal and proximal V_H genes to create a diverse antibody repertoire. It will be important to determine whether such and related epigenetic regulations are functioning properly in systemic autoimmunity.

19.14 Acknowledgement

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References

1. Viau, M. and Zouali, M. (2005) B-lymphocytes, innate immunity, and autoimmunity. *Clinical Immunology* **114**, 17–26.
2. Lim, P.L. and Zouali, M. (2006) Pathogenic autoantibodies: emerging insights into tissue injury. *Immunology Letters* **103**, 17–26.
3. Zouali, M. (2008) B lymphocytes - chief players and therapeutic targets in autoimmune diseases. *Frontiers in Bioscience* **13**, 4852–4861.
4. Nutt, S.L. and Kee, B.L. (2007) The transcriptional regulation of B cell lineage commitment. *Immunity* **26**, 715–725.
5. Delogu, A., Schebesta, A., Sun, Q. *et al.* (2006) Gene repression by Pax5 in B cells is essential for blood cell homeostasis and is reversed in plasma cells. *Immunity* **24**, 269–281.
6. Pongubala, J.M., Northrup, D.L., Lancki, D.W. *et al.* (2008) Transcription factor EBF restricts alternative lineage options and promotes B cell fate commitment independently of Pax5. *Nature Immunology* **9**, 203–215.
7. Buhl, A.M. and Cambier, J.C. (1997) Co-receptor and accessory regulation of B-cell antigen receptor signal transduction. *Immunological Reviews* **160**, 127–138.
8. Hasler, P. and Zouali, M. (2001) B cell receptor signaling and autoimmunity. *FASEB Journal* **15**, 2085–2098.
9. Joazeiro, C.A., Wing, S.S., Huang, H. *et al.* (1999) The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science* **286**, 309–312.
10. Thien, C.B. and Langdon, W.Y. (2005) c-Cbl and Cbl-b ubiquitin ligases: substrate diversity and the negative regulation of signalling responses. *Biochemical Journal* **391**, 153–166.
11. Kitaura, Y., Jang, I.K., Wang, Y. *et al.* (2007) Control of the B cell-intrinsic tolerance programs by ubiquitin ligases Cbl and Cbl-b. *Immunity* **26**, 567–578.
12. Schlissel, M.S. (2003) Regulating antigen-receptor gene assembly. *Nature Reviews Immunology* **3**, 890–899.
13. Brekke, K.M. and Garrard, W.T. (2004) Assembly and analysis of the mouse immunoglobulin kappa gene sequence. *Immunogenetics* **56**, 490–505.

14. Lazorchak, A.S., Schlissel, M.S. and Zhuang, Y. (2006) E2A and IRF-4/Pip promote chromatin modification and transcription of the immunoglobulin kappa locus in pre-B cells. *Molecular and Cellular Biology* **26**, 810–821.
15. Goldmit, M., Ji, Y., Skok, J. *et al.* (2005) Epigenetic ontogeny of the Igk locus during B cell development. *Nature Immunology* **6**, 198–203.
16. Liu, Z., Widlak, P., Zou, Y. *et al.* (2006) A recombination silencer that specifies heterochromatin positioning and ikaros association in the immunoglobulin kappa locus. *Immunity* **24**, 405–415.
17. Medina, K.L., Pongubala, J.M., Reddy, K.L. *et al.* (2004) Assembling a gene regulatory network for specification of the B cell fate. *Developmental Cell* **7**, 607–617.
18. Yu, W., Misulovin, Z., Suh, H. *et al.* (1999) Coordinate regulation of RAG1 and RAG2 by cell type-specific DNA elements 5′ of RAG2. *Science* **285**, 1080–1084.
19. Jung, D., Giallourakis, C., Mostoslavsky, R. and Alt, F.W. (2006) Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. *Annual Review of Immunology* **24**, 541–570.
20. Kosak, S.T., Skok, J.A., Medina, K.L. *et al.* (2002) Subnuclear compartmentalization of immunoglobulin loci during lymphocyte development. *Science* **296**, 158–162.
21. Sayegh, C.E., Jhunjhunwala, S., Riblet, R. and Murre, C. (2005) Visualization of looping involving the immunoglobulin heavy-chain locus in developing B cells. *Genes and Development* **19**, 322–327.
22. Chowdhury, D. and Sen, R. (2001) Stepwise activation of the immunoglobulin mu heavy chain gene locus. *EMBO Journal* **20**, 6394–6403.
23. Bolland, D.J., Wood, A.L., Johnston, C.M. *et al.* (2004) Antisense intergenic transcription in V(D)J recombination. *Nature Immunology* **5**, 630–637.
24. Reynaud, D., Demarco, I.A., Reddy, K.L. *et al.* (2008) Regulation of B cell fate commitment and immunoglobulin heavy-chain gene rearrangements by Ikaros. *Nature Immunology* **9**, 927–936.
25. Smale, S.T. (2003) The establishment and maintenance of lymphocyte identity through gene silencing. *Nature Immunology* **4**, 607–615.
26. Kondrack, R.M., Harbertson, J., Tan, J.T. *et al.* (2003) Interleukin 7 regulates the survival and generation of memory CD4 cells. *Journal of Experimental Medicine* **198**, 1797–1806.
27. Brown, K.E., Baxter, J., Graf, D. *et al.* (1999) Dynamic repositioning of genes in the nucleus of lymphocytes preparing for cell division. *Molecular Cell* **3**, 207–217.
28. Maes, J., O'Neill, L.P., Cavelier, P. *et al.* (2001) Chromatin remodeling at the Ig loci prior to V(D)J recombination. *Journal of Immunology* **167**, 866–874.
29. Morshead, K.B., Ciccone, D.N., Taverna, S.D. *et al.* (2003) Antigen receptor loci poised for V(D)J rearrangement are broadly associated with BRG1 and flanked by peaks of histone H3 dimethylated at lysine 4. *Proceedings of the National Academy of Sciences USA* **100**, 11577–11582.
30. Fuxa, M., Skok, J., Souabni, A. *et al.* (2004) Pax5 induces V-to-DJ rearrangements and locus contraction of the immunoglobulin heavy-chain gene. *Genes and Development* **18**, 411–422.
31. Hewitt, S.L., Farmer, D., Marszalek, K. *et al.* (2008) Association between the Igk and Igh immunoglobulin loci mediated by the 3′ Igk enhancer induces 'decontraction' of the Igh locus in pre-B cells. *Nature Immunology* **9**, 396–404.
32. Chowdhury, D. and Sen, R. (2003) Transient IL-7/IL-7R signaling provides a mechanism for feedback inhibition of immunoglobulin heavy chain gene rearrangements. *Immunity* **18**, 229–241.
33. Spilianakis, C.G., Lalioti, M.D., Town, T. *et al.* (2005) Interchromosomal associations between alternatively expressed loci. *Nature* **435**, 637–645.
34. Bates, J.G., Cado, D., Nolla, H. and Schlissel, M.S. (2007) Chromosomal position of a VH gene segment determines its activation and inactivation as a substrate for V(D)J recombination. *Journal of Experimental Medicine* **204**, 3247–3256.
35. Martin, C. and Zhang, Y. (2005) The diverse functions of histone lysine methylation. *Nature Reviews Molecular & Cell Biology* **6**, 838–849.
36. Su, I.H., Basavaraj, A., Krutchinsky, A.N. *et al.* (2003) Ezh2 controls B cell development through histone H3 methylation and Igh rearrangement. *Nature Immunology* **4**, 124–131.

37. Xu, C.R., Schaffer, L., Head, S.R. and Feeney, A.J. (2008) Reciprocal patterns of methylation of H3K36 and H3K27 on proximal vs. distal IgVH genes are modulated by IL-7 and Pax5. *Proceedings of the National Academy of Sciences USA* **105**, 8685–8690.
38. Hsu, L.Y., Liang, H.E., Johnson, K. *et al.* (2004) Pax5 activates immunoglobulin heavy chain V to DJ rearrangement in transgenic thymocytes. *Journal of Experimental Medicine* **199**, 825–830.
39. Hesslein, D.G., Pflugh, D.L., Chowdhury, D. *et al.* (2003) Pax5 is required for recombination of transcribed, acetylated 5′ IgH V gene segments. *Genes and Development* **17**, 37–42.
40. van Anken, E., Romijn, E.P., Maggioni, C. *et al.* (2003) Sequential waves of functionally related proteins are expressed when B cells prepare for antibody secretion. *Immunity* **18**, 243–253.
41. Zouali, M. (2005) Taming lupus. *Scientific American* **292**, 58–65.
42. Zouali, M., Fournie, G.J. and Theze, J. (1991) Quantitative clonal analysis of the B cell repertoire in human lupus. *Cellular Immunology* **133**, 161–177.
43. Yurasov, S., Tiller, T., Tsuiji, M. *et al.* (2006) Persistent expression of autoantibodies in SLE patients in remission. *Journal of Experimental Medicine* **203**, 2255–2261.
44. Zouali, M. (2002) B cell diversity and longevity in systemic autoimmunity. *Molecular Immunology* **38**, 895–901.
45. Zouali, M. and Sarmay, G. (2004) B lymphocyte signaling pathways in systemic autoimmunity: implications for pathogenesis and treatment. *Arthritis & Rheumatism* **50**, 2730–2741.
46. Chang, N.H., McKenzie, T., Bonventi, G. *et al.* (2008) Expanded population of activated antigen-engaged cells within the naive B cell compartment of patients with systemic lupus erythematosus. *Journal of Immunology* **180**, 1276–1284.
47. Teague, B.N., Pan, Y., Mudd, P.A. *et al.* (2007) Cutting edge: transitional T3 B cells do not give rise to mature B cells, have undergone selection, and are reduced in murine lupus. *Journal of Immunology* **178**, 7511–7515.
48. Bensimon, C., Chastagner, P. and Zouali, M. (1994) Human lupus anti-DNA autoantibodies undergo essentially primary V kappa gene rearrangements. *EMBO Journal* **13**, 2951–2962.
49. Radic, M.Z. and Zouali, M. (1996) Receptor editing, immune diversification, and self-tolerance. *Immunity* **5**, 505–511.
50. Zouali, M. (2008) Receptor editing and receptor revision in rheumatic autoimmune diseases. *Trends in Immunology* **29**, 103–109.
51. Watson, L.C., Moffatt-Blue, C.S., McDonald, R.Z. *et al.* (2006) Paucity of V-D-D-J rearrangements and VH replacement events in lupus prone and nonautoimmune TdT−/− and TdT+/+ mice. *Journal of Immunology* **177**, 1120–1128.
52. Kumar, K.R., Li, L., Yan, M. *et al.* (2006) Regulation of B cell tolerance by the lupus susceptibility gene Ly108. *Science* **312**, 1665–1669.
53. Lamoureux, J.L., Watson, L.C., Cherrier, M. *et al.* (2007) Reduced receptor editing in lupus-prone MRL/lpr mice. *Journal of Experimental Medicine* **204**, 2853–2864.
54. Liu, Y., Li, L., Kumar, K.R. *et al.* (2007) Lupus susceptibility genes may breach tolerance to DNA by impairing receptor editing of nuclear antigen-reactive B cells. *Journal of Immunology* **179**, 1340–1352.
55. Yasuda, S., Stevens, R.L., Terada, T. *et al.* (2007) Defective expression of Ras guanyl nucleotide-releasing protein 1 in a subset of patients with systemic lupus erythematosus. *Journal of Immunology* **179**, 4890–4900.
56. Kuo, C.C. and Lin, S.C. (2007) Altered FOXO1 transcript levels in peripheral blood mononuclear cells of systemic lupus erythematosus and rheumatoid arthritis patients. *Molecular Medicine* **13**, 561–566.
57. Chung, E.Y., Liu, J., Zhang, Y. and Ma, X. (2007) Differential expression in lupus-associated IL-10 promoter single-nucleotide polymorphisms is mediated by poly(ADP-ribose) polymerase-1. *Genes and Immunity* **8**, 577–589.
58. Suzuki, N., Harada, T., Mihara, S. and Sakane, T. (1996) Characterization of a germline Vk gene encoding cationic anti-DNA antibody and role of receptor editing for development of the autoantibody in patients with systemic lupus erythematosus. *Journal of Clinical Investigation* **98**, 1843–1850.

59. Samuels, J., Ng, Y.S., Coupillaud, C. *et al.* (2005) Impaired early B cell tolerance in patients with rheumatoid arthritis. *Journal of Experimental Medicine* **201**, 1659–1667.
60. Yurasov, S., Wardemann, H., Hammersen, J. *et al.* (2005) Defective B cell tolerance checkpoints in systemic lupus erythematosus. *Journal of Experimental Medicine* **201**, 703–711.
61. Faber, C., Morbach, H., Singh, S.K. and Girschick, H.J. (2006) Differential expression patterns of recombination-activating genes in individual mature B cells in juvenile idiopathic arthritis. *Annals of the Rheumatic Diseases* **65**, 1351–1356.
62. Mazari, L., Ouarzane, M. and Zouali, M. (2007) Subversion of B lymphocyte tolerance by hydralazine, a potential mechanism for drug-induced lupus. *Proceedings of the National Academy of Sciences USA* **104**, 6317–6322.
63. Feinberg, A.P. (2007) Phenotypic plasticity and the epigenetics of human disease. *Nature* **447**, 433–440.
64. Brauninger, A., Goossens, T., Rajewsky, K. and Kuppers, R. (2001) Regulation of immunoglobulin light chain gene rearrangements during early B cell development in the human. *European Journal of Immunology* **31**, 3631–3637.
65. Klein, F., Feldhahn, N., Mooster, J.L. *et al.* (2005) Tracing the pre-B to immature B cell transition in human leukemia cells reveals a coordinated sequence of primary and secondary IGK gene rearrangement, IGK deletion, and IGL gene rearrangement. *Journal of Immunology* **174**, 367–375.
66. Vela, J.L., Ait-Azzouzene, D., Duong, B.H. *et al.* (2008) Rearrangement of mouse immunoglobulin kappa deleting element recombining sequence promotes immune tolerance and lambda B cell production. *Immunity* **28**, 161–170.

Abstract

B lymphocytes play a crucial role in the pathogenesis of systemic autoimmune diseases. Despite major advances, the mechanisms that account for disruption of B cell tolerance in systemic autoimmunity remain under scrutiny. Mounting evidence indicates that epigenetic factors shape B cell development and repertoire diversity. They include factors that serve a central function in rearrangement of the immunoglobulin loci by simultaneously regulating the expression of immunoglobulin recombination activating genes, controlling immunoglobulin variable-region gene accessibility and compaction of the immunoglobulin heavy-chain locus, and factors that bind sites in the recombinase locus and regulate histone acetylation, as well as transcription. Other studies have revealed that transcription of immunoglobulin genes located on one chromosome can be controlled by regulatory elements located on a different chromosome. This article focuses on potential epigenetic alterations that may underlie a break of tolerance in the B lymphocyte compartment in autoimmune disease.

Key words: B lymphocytes; lupus; receptor editing; tolerance; rheumatoid arthritis; B cells

PART IV

Towards Novel Epigenetic-Based Immuno-Intervention Strategies in Autoimmune Disease

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Protective effects of epigenetic modifications in experimental inflammatory bowel disease

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20.1 Introduction

Inflammatory bowel disease is a chronic condition, accompanying patients throughout their whole life. Although the pathogenesis has not been definitely clarified, various studies have led to the consensus that, in genetically predisposed individuals, exogenous and host factors together result in a chronic state of dysregulated mucosal immune function that is further modified by specific environmental factors [1].

The two major types of inflammatory bowel disease are ulcerative colitis and Crohn's disease. Whereas ulcerative colitis is limited to the large bowel, Crohn's disease can affect the entire gastrointestinal tract. In contrast to ulcerative colitis where the inflammation is limited to superficial layers of the intestinal wall, Crohn's disease affects the entire bowel wall (transmural inflammation). Although there are additional macroscopic as well as histological differences between the two diseases, it is sometimes impossible to decide whether a patient is suffering from Crohn's disease or ulcerative colitis. In severe disease, suppression of the immune system by classic immunosuppressive agents such as azathioprine and steroids serve as standard treatment. Interestingly, a subset of patients with Crohn's disease and ulcerative colitis respond with a clinical improvement after receiving the neutralizing tumour necrosis factor α (TNF-α) antibody infliximab or adalimumab, respectively. The disadvantage of this treatment is the long half-life of the antibodies. In addition, a significant subset of patients is primarily resistant to this treatment or develops a secondary resistance during the

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following disease course. Thus, the search for novel therapeutic options represents a key interest of a number of research groups worldwide [2].

20.2 Mechanisms of protein acetylation and deacetylation

Over the last few years, epigenetic modifications on gene expression have emerged as a focus of basic as well as medical research, in particular to develop novel pharmacological compounds or diagnostic tools. The modification of histone-acetylation patterns in particular seems to represent a promising new therapeutic strategy to modulate cell functions in autoimmune diseases or cancer.

To investigate and possibly regulate the cellular effects of these modifications, it is crucial to understand the molecular mechanisms of histone acetylation and deacetylation. The N-terminal ends of histones are positively charged, allowing an interaction with the negatively charged phosphate groups of the DNA backbone (Figure 20.1). Histone acetylation neutralizes the positive charges of the histone tails, thus weakening the bindings to the DNA. Accordingly, acetylated histones represent the decondensed, 'open' state of chromatin, where the DNA is accessible for transcription factors [3].

Two classes of enzymes account for the process of histone acetylation and deacetylation. Histone acetylation is mediated by histone acetyltransferases (HATs), a group of enzymes that acetylates lysine amino acids of histones by transferring an acetyl group from acetyl-CoA. In contrast, histone deacetylases (HDACs) remove acetyl groups from the histone tails and restore their positive charge, resulting in a high-affinity binding between histones and DNA [4] (Figure 20.1). This deacetylation results in a 'closed' state of the chromatin, where DNA-binding sites are inaccessible for transcription factors [5]. This condition is consequently followed by a decrease in gene expression.

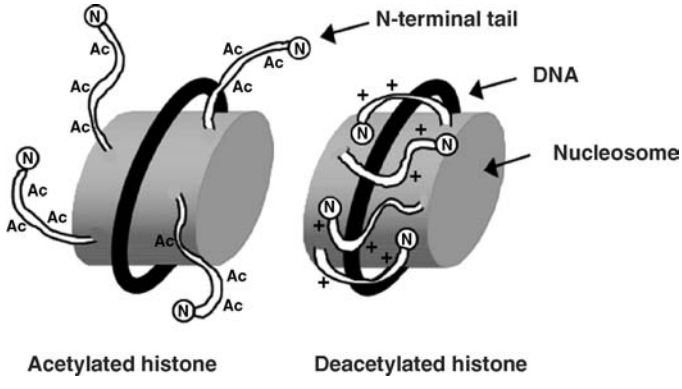

Figure 20.1 Schematic representation of acetylated and deacetylated histones. The N-terminal ends of the histones in their deacetylated state are positively charged and adhere to the negatively charged DNA (right). Acetylated (Ac) histones are neutralized and the DNA is accessible for transcription factors (left).

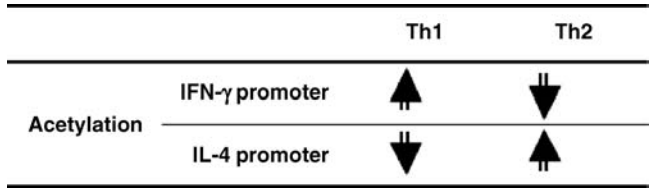

Figure 20.2 Schematic representation of the differential acetylation patterns during Th1/Th2 helper cell polarization. The arrows indicate promoter acetylation at day 6 after stimulation under polarizing conditions (Th1: IL-12 and anti-IL-4; Th2: IL-4, anti-IFN-γ and anti-IL-12) [6].

Taken together, histone acetylation plays an important role in the regulation of transcription and gene expression. Acetylated chromatin is accessible and deacetylated chromatin is inaccessible for the binding of transcription factors and subsequent gene expression. One example is provided by polarized T helper (Th) cells that exhibit a characteristic acetylation pattern of key cytokine promoter regions. Thus, in Th1 cells the histones localized at the site of the interferon γ (IFN-γ) promoter and in Th2 cells the histones localized at the interleukin (IL)-4 promoter region are hyperacetylated [6] (Figure 20.2).

Not only histones represent targets of HATs and HDACs: more than 50 other non-histone proteins have been identified whose function is regulated by acetylation and deacetylation [7]. They include well-characterized transcription factors, such as hypoxia-inducible factor-1α (HIF1α), GATA-binding protein 3 (GATA3), nuclear factor κB (NF-κB) subunit p65, signal transducers and activators of transcription 3 (STAT3) and SMAD7 (mothers against decapentaplegic homologues), as well as the structural protein α-tubulin.

Eighteen different HDACs have been described in humans, which can be assigned to four classes based on sequence homology to yeast HDACs [8,9] (Table 20.1). Whereas class I HDACs are expressed constitutively and are localized in the nucleus, members of class II are inducible in a tissue-dependent manner and can be found not only in the nucleus but also in the cytoplasm. Their activity can be inhibited by different pharmacological groups of HDAC inhibitors (HDIs) that can be assigned to four classes depending on their structural characteristics [10] (Table 20.2). The majority of HDIs exert their effect rather unspecifically on zinc-based HDACs. There are exceptions, however, such as MS-275, which has been characterized as a specific inhibitor of HDAC1 [11]. One would expect that the presence of HDIs is followed by global changes in gene transcription and protein modification. However, less than 10% of genes have been shown to be differentially expressed in cells treated with HDIs [12–14].

Recently HDIs have been evaluated in clinical trials as anti-cancer agents [7]. The effects depend on the HDI concentration, exposure time, cell type and structure. Inhibition of HDAC induces cycle arrest, apoptosis and terminal differentiation of transformed cells. These pro-apoptotic and anti-proliferative properties of this pharmacological class suggest their potential use as novel therapeutic drugs to treat malignancies. Consequently there are several HDIs currently in clinical trials against

Table 20.1 Classes of HDAC.

Class	Examples	Characteristics
Class I	HDAC1, HDAC2, HDAC3, HDAC8	• 22–55 kDa • Related to yeast RPD3 deacetylase
Class II	HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, HDAC10	• Share homology with yeast HDA1 deacetylase • Larger molecules: 120–135 kDa
Class III	Sirtuins (SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, SIRT7)	• Sirtuins appear not to have histones as their primary substrate • Not affected by the standard HDIs • Under investigation for their effect on aging processes and degenerative diseases
Class IV	HDAC11	• HDAC 11 represents its own class Shares characteristics with classes I and II

Table 20.2 Classes of HDI.

Class	Examples
Hydroxamic acids	Suberyolanilide hydroxamic acid, ITF2357, trichostatin A
Cyclic peptides	Apicidine
Benzamides	MS-275
Short-chain fatty acids	Valproic acid, butyrate

various types of solid tumour and haematological cancer [15,16]. In addition to these studies, there is increasing evidence for an additional anti-inflammatory potency of HDIs [17–20].

20.3 Anti-inflammatory effect of epigenetic modifications *in vitro*

Recent data from our own group indicate that HDIs, independent of their class, exert an anti-inflammatory potency via suppression of pro-inflammatory cytokines [17]. These anti-inflammatory properties were demonstrated *in vitro* by suppression of pro-inflammatory cytokines such as IL-1β, IFN-γ or TNF-α in lipopolysaccharide (LPS)- or cytokine-stimulated human peripheral blood mononuclear cells (Figure 20.3).

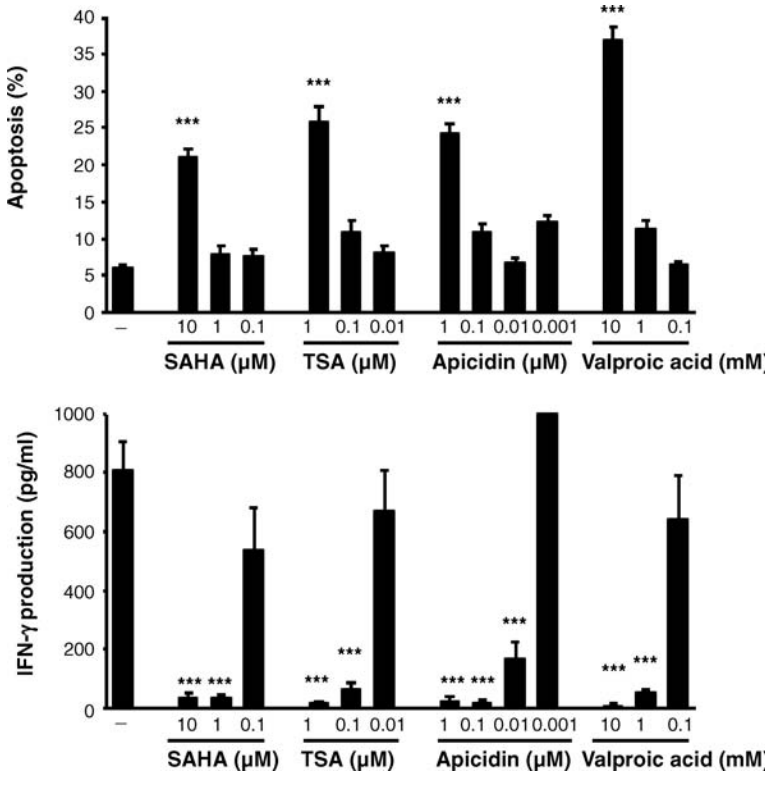

Figure 20.3 Anti-inflammatory properties of HDIs. Peripheral blood mononuclear cells were isolated from normal human blood and stimulated with concanavalin A for 24 h. Trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), apicidin and valproic acid were added 1 h before the stimulation. Apoptosis induction was assessed by annexin V/propidium iodide staining and flow cytometry analysis, IFN-γ production was determined by ELISA ($n = 6$).

One could argue that the reduced production of pro-inflammatory cytokines is the result of apoptosis induction by the HDIs. However, the suppression of pro-inflammatory cytokines by the HDIs occurs at significantly lower concentrations than those required for apoptosis induction (Figure 20.3).

These anti-inflammatory properties could be confirmed by our group for various HDIs, independent of their structural class, in different human and murine cell types. Agents included in these studies were suberyolanilide hydroxamic acid (SAHA), ITF2357, valproic acid, trichostatin A and apicidin. They all suppress pro-inflammatory cytokines. However, they differ significantly in their effective active concentration (e.g. SAHA is 1000 times more effective than valproic acid). Although the precise mechanisms leading to this anti-inflammatory efficacy are still unknown, several studies offer possible explanations. For instance, the differentiation of Th cells is paralleled by a change in the acetylation pattern at key promoter sites [6] (Figure 20.1). Hence,

transcription of the respective genes might be directly modulated by a change in the acetylation pattern of the related promoter region.

Recently, the group of Hancock demonstrated that the activation state of the transcription factor Foxp3 is directly affected by the HDI trichostatin A in a murine model [21]. Foxp3 represents a key marker for regulatory T cells, which are currently considered as the critical suppressive cell type in the control of inflammatory responses. The activation of Foxp3 itself depends on its acetylation state and is regulated by HDAC7 and HDAC9. Tao *et al.* reported a 'boosting' effect of an HDI on $Foxp3^+$ regulatory T cells [21]. In parallel, HDAC9-knockout mice were protected in a model of experimental colitis mediated by an upregulation of regulatory T cells [21]. These findings are even more important considering the fact that there are very few reported effects of HDIs that result in activation rather than downregulation of cells [21].

The effect of HDIs on the acetylation state of p65, a subunit of the transcription factor NF-κB, is controversial [22–27]. p65 is acetylated in the nucleus by the HAT p300 and deacetylated by HDAC3 [28]. Blocking this deacetylation step should keep the activated p65 in the nucleus, and result in hyperactivation of NF-κB. Although some studies [26,27] have confirmed this effect, others have reported the opposite: in these studies NF-κB activation was reduced and the translocation of p65 into the nucleus was inhibited [22,23]. The conclusion drawn from these studies was that SAHA affects the NF-κB signal transduction pathway upstream of the separation of IκB and the p50/p65 complex [29] (Figure 20.4, pathways a and c). Importantly, inhibition of HDAC was

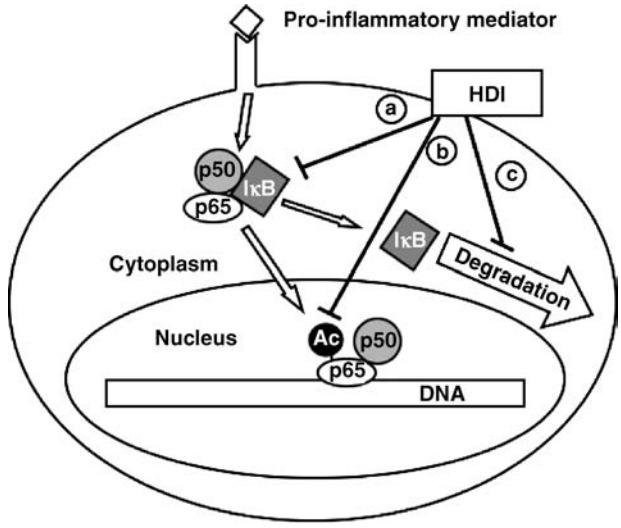

Figure 20.4 Impact of HDIs on the NF-κB pathway. HDIs are able to impact NF-κB (p50 + p65) activation in different ways. An inhibition of inhibitor of κB (IκB) binding (a) leads to increased NF-κB translocation into the nucleus, therefore enhancing NF-κB activity. An inhibition of IκB degradation (c) enhances the probability of IκB binding to NF-κB, leading to an impaired NF-κB activity. In the nucleus (b) HDAC inhibition prevents NF-κB deactivation.

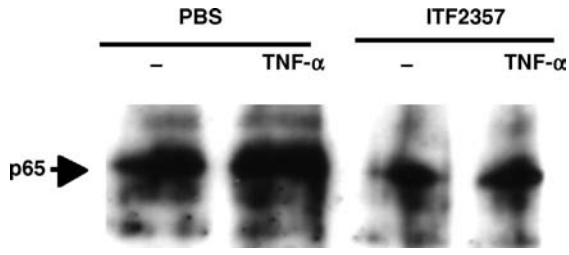

Figure 20.5 Reduced nuclear translocation of p65 in cells treated with the HDI ITF2357. HeLa cells were pretreated with ITF2357 (1 μM) for 1 h, and subsequently stimulated with TNF-α for 40 min. Nuclear extracts were analysed for p65 by Western blotting.

found to suppress phosphorylation and nuclear translocation of p65 in the nucleus (Figure 20.4, pathway b), an effect our group could confirm, demonstrating a reduced amount of p65 in the nucleus after treatment with SAHA and ITF2357 *in vitro* as well as for the first time *in vivo* [18] (Figure 20.5).

20.4 Impact of HDAC inhibition in models of experimental colitis

The anti-inflammatory efficacy of HDAC could be demonstrated in various *in vivo* models including concanavalin A-induced hepatitis, LPS-induced shock, experimental autoimmune encephalomyelitis and various models of experimental colitis [17,19,20,30]. As outlined in section 20.1, inflammatory bowel disease is a chronic disease accompanying patients throughout their life. The treatment options and understanding of the pathogenesis of the disease have improved dramatically over recent years. There is still a significant subgroup of patients for whom an appropriate treatment is missing. The profound inhibition of pro-inflammatory cytokines *in vitro* as well as in the above-mentioned *in vivo* models suggest that HDI treatment might represent an attractive novel pharmacological approach for the therapy of inflammatory bowel disease. Thus, we decided to evaluate several HDI in models of experimental colitis in mice.

There are several models of chemically induced experimental colitis available, each reflecting specific characteristics of the human disease. The model of acute dextran sulphate sodium (DSS)-induced colitis is well-known and often used to evaluate novel pharmaceutical compounds for their anti-inflammatory capacity [31]. However, this model is not considered to be an immunological model of inflammatory bowel diseases because during the acute disease course the epithelial barrier is disrupted, resulting in an inflammatory response of the mucosa. Yet, stimulation of a T cell response occurs and represents characteristics of the human disease, indicating its uselfulness as a model of chronic DSS-induced colitis. On the other hand, the models of trinitrobenzene- and oxazolone-induced colitis represent Th1- and Th2-mediated models of colitis, respectively [32,33].

In general, the anti-inflammatory potency of HDIs was evaluated in different models of colitis [17]. Our data indicated that HDIs of different classes could ameliorate the symptoms of acute DSS-induced colitis. Weight loss, bleeding and colon shortening could be reduced using SAHA, ITF2357 and the short chain fatty acid valproic acid. These macroscopic data were paralleled by a reduction of pro-inflammatory cytokines at the site of inflammation in colon cultures and further underlined by a reduced histological inflammation score. Figure 20.6 shows data from one acute DSS-induced colitis experiment using SAHA as the HDI. SAHA treatment resulted in a reduced weight loss and a decrease in macroscopic mucosal inflammation as indicated by the endoscopic pictures (Figure 20.6b). The effect on trinitrobenzene-induced colitis was very similar. Even though an ameliorating effect of all HDIs was observed in both models, SAHA and ITF2357 were superior to valproic acid.

Apoptosis induction mediated by the inhibition of HDAC might contribute to the therapeutic efficacy in these models. For example in healthy humans, lamina propria mononuclear cells exhibit a high susceptibility to Fas-mediated apoptosis, whereas lamina propria mononuclear cells from patients with Crohn's disease have been

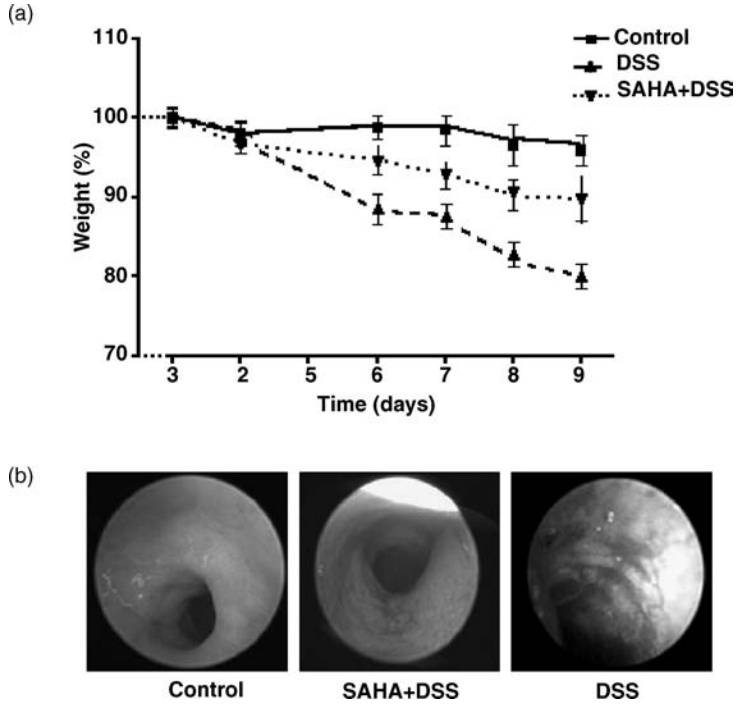

Figure 20.6 Effect of SAHA treatment on acute DSS-induced colitis. Mice ($n = 7$) were exposed to DSS (4%) in drinking water, and SAHA or vehicle were administered (50 mg/kg of body weight) via oral gavage. (a) The normalized weight curves from days 3 to 9. On day 7 the mice were examined via lower endoscopy (b). The DSS-treated animals show a clear reduction of inflammation compared to the untreated mice.

shown to be resistant to multiple apoptotic pathways [34]. Here, treatment with the anti-TNF-α antibody infliximab resulted in monocyte- and caspase-3-dependent T cell apoptosis [35]. In our models, HDI treatment was followed by a significant increase in lamina propria mononuclear cell apoptosis, which might contribute to the described anti-inflammatory efficacy.

Similar results were obtained by another group with trichostatin A in the DSS-induced colitis model. Here the authors explained the beneficial effect with an enhanced functionality of regulatory T cells [21]. In addition, they could mimic the HDI effect using HDAC9-knockout mice, thus suggesting a critical role of HDAC9 in the function of regulatory T cells. The authors do not claim to have identified the only relevant target for the HDI, but the data clearly demonstrate a predominant role of the regulatory T cells in these models. When considering HDIs as novel possible therapeutic target for the treatment of inflammatory bowel disease their apoptosis-inducing property is of particular interest. This is based on the clinical observation that ulcerative colitis is associated with an increased risk of developing colon cancer after a disease course of more than 10 years in patients suffering from pan-colitis [36].

These clinical observations are underlined by experimental data characterizing the key inflammatory transcription factor NF-κB as a link between inflammation and carcinogenesis [37,38]. These studies were performed in models of inflammation-associated colon cancer. Here, in the absence of NF-κB activation in colon epithelial cells, tumour development was clearly suppressed, while no effect on inflammation was observed. In contrast, when NF-κB activation was abrogated in macrophages of the lamina propria, suppression of tumour development was paralleled with a reduction in intestinal inflammation.

Given the prominent anti-inflammatory effect in the models of experimental colitis as well as the well-known anti-proliferative capacity of HDIs, one would assume a strong tumour-preventing capacity of HDAC inhibition in models of inflammation-induced tumorigenesis. In this regard, there are several models of inflammation-associated cancerogenesis, mediated by chronic colitis, where tumours develop at the site of inflammation. The azoxymethane-/DSS-induced model is based on a single injection of the carcinogen azoxymethane followed by induction of a chronic DSS-induced colitis (Figure 20.7a). The development from initial dysplasia to full-grown adenomas can be monitored by lower endoscopy in the living mice (Figure 20.7c). In the azoxymethane-/DSS-induced model continuous treatment with the HDIs SAHA and ITF2357 attenuated both tumour growth and development. Again, ITF2357, as seen in the colitis models, was more potent than SAHA [18].

Another model of inflammation-mediated tumorigenesis is the IL-10-knockout model. These mice spontaneously develop colitis and, with age, dysplasia can progress to infiltrating carcinomas. This progression of tumour development can be enhanced or accelerated by a 5-day treatment with the cyclooxygenase-2 inhibitor celecoxib [39]. Despite the fact that the administration of HDIs did not start until the age of 12 weeks, HDI treatment was associated with an inhibition of tumorigenesis in the following 4 weeks. Hence, treatment with the HDI ITF2357 was highly effective in this therapeutic model of inflammation-mediated carcinogenesis.

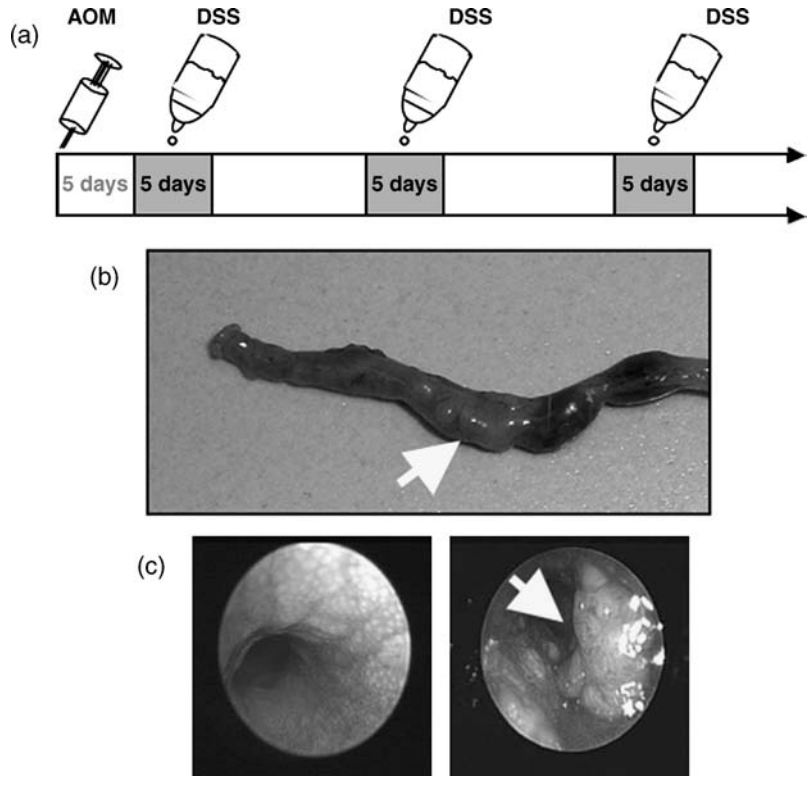

Figure 20.7 Azoxymethane (AOM)/DSS-induced model of inflammation-mediated colon cancerogenesis. (a) A single injection of the carinogen azoxymethane (12.5 mg/kg of body weight) was followed by the induction of chronic colitis by repeated low-dose treatments (5 days) with DSS (3%) in drinking water. (b) The distal part of the colon at day 50, displaying the prominent presence of adenoma (arrow). (c) The mice were continuously monitored via lower endoscopy. Shown are images of the colon of a control mouse (left) and an azoxymethane-/DSS-treated mouse (right) at day 40. The arrow indicates prominent adenomas.

20.5 Perspectives

For several years, HDIs were of interest solely for researchers working in oncology. Currently, the focus has changed to the anti-inflammatory impact of these agents [40]. Several experimental models have revealed the anti-inflammatory effects of HDIs, including LPS-induced shock, concanavalin A-induced hepatitis [20], murine models of systemic lupus erythematosus [30], arthritis [41] and ovalbumin-induced airway inflammation [42]. Our own data in models of experimental colitis further support the concept that inhibition of HDAC is associated with an anti-inflammatory effect.

Yet the exact mechanisms behind these anti-inflammatory properties are not completely solved. In most experimental set-ups it is hard to determine whether

histone- or non-histone-mediated effects are more important, whether gene induction or suppression is the crucial factor, or whether apoptosis induction and cell toxicity imitate anti-inflammatory effects. For the majority of the HDIs, the target HDACs remain unknown. At this point, two possible explanations seem to contribute to the therapeutic effects observed *in vivo*: an inhibitory effect on the NF-κB pathway [18] and an enhancing effect on regulatory T cells mediated by HDAC9 [21].

In addition to the unclear mode of action behind the beneficial effects observed in the disease models described, it has to be pointed out that HDIs are cytotoxic agents and induce cell-cycle arrest and apoptosis, which might represent a risk during long-term treatments [43]. New insights into the roles of the distinct HDAC in the different cell types will help to improve therapeutic strategies in chronic inflammation and, in parallel, reduce potential side effects. Nevertheless in a medium-term perspective, treatment with HDIs is a promising therapeutic strategy for chronic inflammation in general as well as chronic intestinal inflammation and associated tumour development in particular.

References

1. Xavier, R.J. and Podolsky, D.K. (2007) Unravelling the pathogenesis of inflammatory bowel disease. *Nature* **448**, 427–434.
2. Lichtenstein, G.R., Abreu, M.T., Cohen, R. *et al.* (2006) American Gastroenterological Association Institute technical review on corticosteroids, immunomodulators, and infliximab in inflammatory bowel disease. *Gastroenterology* **130**, 940–987.
3. Urnov, F.D. and Wolffe, A.P. (2001) Above and within the genome: epigenetics past and present. *Journal of Mammary Gland Biology and Neoplasia* **6**, 153–167.
4. Roth, S.Y., Denu, J.M. and Allis, C.D. (2001) Histone acetyltransferases. *Annual Review of Biochemistry* **70**, 81–120.
5. Eggert, H., Gortchakov, A. and Saumweber, H. (2004) Identification of the *Drosophila* interband-specific protein Z4 as a DNA-binding zinc-finger protein determining chromosomal structure. *Journal of Cell Science* **117**, 4253–4264.
6. Fields, P.E., Kim, S.T. and Flavell, R.A. (2002) Cutting edge: changes in histone acetylation at the IL-4 and IFN-gamma loci accompany Th1/Th2 differentiation. *Journal of Immunology* **169**, 647–650.
7. Xu, W.S., Parmigiani, R.B. and Marks, P.A. (2007) Histone deacetylase inhibitors: molecular mechanisms of action. *Oncogene* **26**, 5541–5552.
8. Dokmanovic, M. and Marks, P.A. (2005) Prospects: histone deacetylase inhibitors. *Journal of Cellular Biochemistry* **96**, 293–304.
9. Marks, P.A. and Dokmanovic, M. (2005) Histone deacetylase inhibitors: discovery and development as anticancer agents. *Expert Opinion on Investigational Drugs* **14**, 1497–1511.
10. Miller, T.A., Witter, D.J. and Belvedere, S. (2003) Histone deacetylase inhibitors. *Journal of Medicinal Chemistry* **46**, 5097–5116.
11. Hu, E., Dul, E., Sung, C.M. *et al.* (2003) Identification of novel isoform-selective inhibitors within class I histone deacetylases. *Journal of Pharmacology and Experimental Therapeutics* **307**, 720–728.
12. Mitsiades, C.S., Mitsiades, N.S., McMullan, C.J. *et al.* (2004) Transcriptional signature of histone deacetylase inhibition in multiple myeloma: biological and clinical implications. *Proceedings of the National Academy of Sciences USA* **101**, 540–545.

13. Glaser, K.B., Staver, M.J., Waring, J.F. *et al.* (2003) Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. *Molecular Cancer Therapeutics* **2**, 151–163.
14. Sasakawa, Y., Naoe, Y., Sogo, N. *et al.* (2005) Marker genes to predict sensitivity to FK228, a histone deacetylase inhibitor. *Biochemical Pharmacology* **69**, 603–616.
15. Marks, P.A. and Breslow, R. (2007) Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug. *Nature Biotechnology* **25**, 84–90.
16. Bolden, J.E., Peart, M.J. and Johnstone, R.W. (2006) Anticancer activities of histone deacetylase inhibitors. *Nature Reviews. Drug Discovery* **5**, 769–784.
17. Glauben, R., Batra, A., Fedke, I. *et al.* (2006) Histone hyperacetylation is associated with amelioration of experimental colitis in mice. *Journal of Immunology* **176**, 5015–5022.
18. Glauben, R., Batra, A., Stroh, T. *et al.* (2008) Histone deacetylases: novel targets for prevention of colitis-associated cancer in mice. *Gut* **57**(5), 613–622.
19. Mishra, N., Reilly, C.M., Brown, D.R. *et al.* (2003) Histone deacetylase inhibitors modulate renal disease in the MRL-lpr/lpr mouse. *Journal of Clinical Investigation* **111**, 539–552.
20. Leoni, F., Fossati, G., Lewis, E.C. *et al.* (2005) The histone deacetylase inhibitor ITF2357 reduces production of pro-inflammatory cytokines in vitro and systemic inflammation in vivo. *Molecular Medicine* **11**, 1–15.
21. Tao, R., de Zoeten, E.F., Ozkaynak, E. *et al.* (2007) Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nature Medicine* **13**, 1299–1307.
22. Ashburner, B.P., Westerheide, S.D. and Baldwin, Jr, A.S. (2001) The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. *Molecular and Cellular Biology* **21**, 7065–7077.
23. Imre, G., Gekeler, V., Leja, A. *et al.* (2006) Histone deacetylase inhibitors suppress the inducibility of nuclear factor-kappaB by tumor necrosis factor-alpha receptor-1 down-regulation. *Cancer Research* **66**, 5409–5418.
24. Liu, H., Chen, Y., Cui, G. *et al.* (2006) Trichostatin A regulates hGCN5 expression and cell cycle on daudi cells in vitro. *Journal of Huazhong University of Science and Technology. Medical Sciences* **26**, 534–537.
25. Takada, Y., Gillenwater, A., Ichikawa, H. *et al.* (2006) Suberoylanilide hydroxamic acid potentiates apoptosis, inhibits invasion, and abolishes osteoclastogenesis by suppressing nuclear factor-kappaB activation. *Journal of Biological Chemistry* **281**, 5612–5622.
26. Dai, Y., Rahmani, M., Dent, P. and Grant, S. (2005) Blockade of histone deacetylase inhibitor-induced RelA/p65 acetylation and NF-kappaB activation potentiates apoptosis in leukemia cells through a process mediated by oxidative damage, XIAP downregulation, and c-Jun N-terminal kinase 1 activation. *Molecular and Cellular Biology* **25**(13), 5429–5444.
27. Quivy, V. and Van Lint, C. (2004) Regulation at multiple levels of NF-kappaB-mediated transactivation by protein acetylation. *Biochemical Pharmacology* **68**(6), 1221–1229.
28. Chen, L., Fischle, W., Verdin, E. *et al.* (2001) Duration of nuclear NF-kappaB action regulated by reversible acetylation. *Science* **293**, 1653–1657.
29. Blanchard, F. and Chipoy, C. (2005) Histone deacetylase inhibitors: new drugs for the treatment of inflammatory diseases? *Drug Discovery Today* **10**, 197–204.
30. Camelo, S., Iglesias, A.H., Hwang, D. *et al.* (2005) Transcriptional therapy with the histone deacetylase inhibitor trichostatin A ameliorates experimental autoimmune encephalomyelitis. *Journal of Neuroimmunology* **164**, 10–21.
31. Olson, A.D., DelBuono, E.A., Bitar, K.N. *et al.* (1995) Antiserum to tumor necrosis factor and failure to prevent murine colitis. *Journal of Pediatric Gastroenterology and Nutrition* **21**, 410–418.
32. Neurath, M.F., Fuss, I., Kelsall, B.L. *et al.* (1995) Antibodies to interleukin 12 abrogate established experimental colitis in mice. *Journal of Experimental Medicine* **182**, 1281–1290.

33. Boirivant, M., Fuss, I.J., Chu, A. *et al.* (1998) Oxazolone colitis: a murine model of T helper cell type 2 colitis treatable with antibodies to interleukin 4. *Journal of Experimental Medicine* **188**, 1929–1939.
34. Neurath, M.F., Finotto, S., Fuss, I. *et al.* (2001) Regulation of T-cell apoptosis in inflammatory bowel disease: to die or not to die, that is the mucosal question. *Trends in Immunology* **22**, 21–26.
35. Lugering, A., Schmidt, M., Lugering, N. *et al.* (2001) Infliximab induces apoptosis in monocytes from patients with chronic active Crohn's disease by using a caspase-dependent pathway. *Gastroenterology* **121**, 1145–1157.
36. Eaden, J.A., Abrams, K.R. and Mayberry, J.F. (2001) The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* **48**, 526–535.
37. Greten, F.R., Eckmann, L., Greten, T.F. *et al.* (2004) IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell* **118**, 285–296.
38. Karin, M. and Greten, F.R. (2005) NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nature Reviews Immunology* **5**, 749–759.
39. Hegazi, R.A., Mady, H.H., Melhem, M.F. *et al.* (2003) Celecoxib and rofecoxib potentiate chronic colitis and premalignant changes in interleukin 10 knockout mice. *Inflammatory Bowel Diseases* **9**, 230–236.
40. Adcock, I.M. (2007) HDAC inhibitors as anti-inflammatory agents. *British Journal of Pharmacology* **150**, 829–831.
41. Chung, Y.L., Lee, M.Y., Wang, A.J. *et al.* (2003) A therapeutic strategy uses histone deacetylase inhibitors to modulate the expression of genes involved in the pathogenesis of rheumatoid arthritis. *Molecular Therapy* **8**, 707–717.
42. Choi, J.H., Oh, S.W., Kang, M.S. *et al.* (2005) Trichostatin A attenuates airway inflammation in mouse asthma model. *Clinical and Experimental Allergy* **35**, 89–96.
43. Marks, P.A., Richon, V.M., Kelly, W.K. *et al.* (2004) Histone deacetylase inhibitors: development as cancer therapy. *Novartis Foundation Symposium* **259**, 269–281; discussion 281–288.

Abstract

Histone deacetylase (HDAC) inhibitors were described initially for their anti-proliferative effect in cancer cell lines as well as for their reduction of experimental tumour growth *in vivo*. Furthermore, several HDAC inhibitors (HDIs) are currently in clinical trials for a variety of solid and haematological cancers. In addition, recent studies provide evidence for an anti-inflammatory potency of HDI *in vitro* and *in vivo* in various models of experimental colitis, as well as colitis-associated tumorigenesis. Still, the exact mechanisms behind these anti-inflammatory properties of HDI are not completely solved. Currently, several possible modes of action are under investigation; for example, the impact on regulatory T cells or on the nuclear factor κB pathway. Inhibition of HDACs might provide a novel strategy in chronic inflammatory disorders such as inflammatory bowel disease, where chronic inflammation is linked to an increased risk for colon cancer.

Key words: histone; acetylation; colitis; IBD; NF-κB; Th cells; HDAC; HDI; epigenetics; inflammation.

21

Epigenetic regulation of autoimmune diseases through deacetylase inhibition

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21.1 Introduction

Autoimmune diseases can be considered a result of a dysfunctional immune system attacking self-antigens. About 5% of the population suffers from autoimmune diseases [1]. Regulatory T cells (Tregs) play an essential role in immunological tolerance to self-antigens [2]. The compromise of Treg functions contributes to the pathogenesis of various autoimmune diseases [3]. Therapeutically modulating or adoptively transferring Tregs has been broadly proposed as an advanced tool to treat autoimmune disorders [4].

Abnormal epigenetic changes may alter the functional regulation of the immune system, leading to human diseases including autoimmune disorders [5,6]. Epigenetic studies generally focus on the mitotic and/or meiotic heritable messages carried by DNA methylation and chromatin structure that are 'memorizable' and can be passed on through generations [7]. More recently, the study of epigenetics has also been extended to new fields such as RNA-interference-mediated heterochromatin formation, histone variants exchange, histone modification and chromatin remodelling [7,8].

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Histone deacetylases (HDACs) or lysine deacetylases remove the acetyl group from the side-chain ε-amino groups of the lysine residue in either histone or non-histone proteins [9]. Most histone-modification enzymes are also actively involved in the post-translational modification of non-histone proteins [10]. To date, more than 70 non-histone proteins have been found to be acetylated *in vivo* [9]. Lysine acetylation and deacetylation function as a molecular switch to control gene transcription and protein function. Lysine acetylation modifies chromatin accessibility and the function of non-histone proteins including transcription factors [11] and cell-surface receptors [12]. Various dietary or synthesized HDAC inhibitors have recently been used to treat autoimmune diseases including rheumatoid arthritis [13], systemic lupus erythematosus [14–17] and experimental autoimmune encephalomyelitis [18]. Epigenetic studies including DNA methylation [19] and epigenome sequencing [20] have been reviewed extensively elsewhere. Here we summarize recent work on treating autoimmune disease by HDAC inhibitors. We emphasize recent progress in modulating Treg function through deacetylase inhibition.

21.2 Regulatory T cells

Tregs play a critical role in controlling the immune response to self-antigens and pathogens via multiple non-overlapping mechanisms [21,22]. Several sets of Tregs exist. These include forkhead box protein P3 (FOXP3) expressing $CD4^+CD25^+$ natural Tregs (nTreg) that originally develop in the thymus [23], transforming growth factor β (TGF-β)-induced $FOXP3^+$ Tregs (iTregs) in the periphery [24–26], interleukin (IL) 10-secreting type 1 regulatory T (Tr1) cells [27], T helper type 3 (Th3) TGF-β-secreting regulatory cells [28] and Qa-1-restricted $CD8^+$ Tregs [29].

The forkhead-family transcription factor FOXP3 was recently identified as a marker of nTregs [30]. More importantly, FOXP3 is also both necessary and sufficient to convert naïve T cells to functional Tregs in mice and in humans [31], although the transcriptional signature of the development and function of nTregs may include both FOXP3-dependent and FOXP3-independent transcriptional events [32,33].

$FOXP3^+$ Treg cells suppress the activation, proliferation and function of various immune cells including $CD4^+$ effector T cells, $CD8^+$ cytotoxic T cells, B cells, dendritic cells and macrophages [34]. A growing list of potential mediators of immunosupression by $FOXP3^+$ Tregs has been identified, which include cell-surface receptors such as lymphocyte-activation gene 3 (LAG-3), glucocorticoid-induced tumour necrosis factor receptor (GITR) and cytotoxic T lymphocyte protein 4 (CTLA-4); secreted suppressive cytokines such as TGF-β1, IL-10, interferon γ and IL-35; enzymes such as granzyme B and haem oxygenase-1; CD39- and CD73-mediated production of adenosine and metabolites such as cAMP. Thus, understanding the mechanisms underlying FOXP3 expression and function is critical to therapeutically modulating Treg function for autoimmune disease.

21.3 Epigenetic regulation of *FOXP3* expression

Murine *Foxp3* was initially identified as a gene specifically expressed in $CD4^+CD25^+$ nTregs but not in other immune cells. Ectopically expressing *Foxp3* in murine $CD4^+$ $CD25^-$ effector T cells could convert these cells to be functionally suppressive [23]. More recently, *FOXP3* expression has also been broadly found in various epithelial cells including breast, lung respiratory and prostate epithelial cells [35], and tumour cells [36,37]. The induction of *FOXP3* expression in murine and human Tregs has been studied extensively in recent years [38].

Many factors, including cytokines such as IL-2 and TGF-β, have been identified as the inducers of murine *Foxp3* gene expression [38,39]. Detailed analyses of the *Foxp3* promoter and enhancer regions have provided molecular clues on the modulation of murine *Foxp3* transcription by these extracellular factors. Consensus binding sites for various key transcription factors, including signal transducer and activator of transcription (STAT) 3 and STAT5, nuclear factor of activated T cells (NFAT), activator protein 1 (AP-1), specificity protein 1 (SP1) and cAMP-response-element-binding protein (CREB)/activating transcription factor (ATF) basic leucine zipper domain (bZIP) transcription factor (CREB/ATF), have been identified on the *Foxp3* promoter [38]. Two enhancers regulating murine *Foxp3* gene expression have been identified recently [40]. The first enhancer maps to the region from $+2079$ to $+2198$ and the second from $+4054$ to $+5216$ in the murine *Foxp3* gene. Both enhancers are associated with NFAT and Smad3, which play a key role in inducing murine *Foxp3* gene expression.

The second enhancer, which was also independently identified by another group, contains STAT5 and CREB/ATF-binding sites [41]. The synergistic cooperation of transcription factors activated by extracellular stimuli, including T cell receptor and other co-stimulation signals, may promote histone H4 acetylation in a site-specific manner, leading to chromatin remodelling and murine *Foxp3* transcription. Consistent with this notion, treatment with the HDAC inhibitor promotes histone acetylation and murine *Foxp3* expression in Tregs [42]. Moreover, the induction of *FOXP3* expression may depend on the DNA methylation status of the *FOXP3* gene as well, since 5-aza-2′-deoxycytidine treatment increases *FOXP3* expression [43].

21.4 FOXP3 acetylation and function

Whereas initially it was thought that molecules such as histone acetyltransferases and HDACs affect only histones, many proteins including transcription factors are now found to be modulated by acetylation and deacetylation. Histone acetylation, which takes place on the N-terminal tails of histones H3 and H4, is generally thought to increase the accessibility of transcriptional machinery to alter gene transcription [44] and is mediated by histone acetyltransferases. For instance, histone acetylatransferase

HIV-1 Tat-interacting protein, 60 kDa (TIP60) can directly acetylate certain transcriptional proteins, such as p53. TIP60 has been found to associate with class II HDACs, such as HDAC7. Under basal conditions the major pools of TIP60 and HDAC7 reside in the nucleus, but the complex undergoes translocation from the nucleus to the cytoplasm [45]. HDAC7 is also able to affect central T cell unresponsiveness by inhibiting T cell death [46].

The dogma is that acetylation of histones is generally linked to opening chromatin, making the nucleosome more accessible to transcripton factors and active transcription [47]. Deacetylation of histones is otherwise associated with condensed chromatin or heterochromatin, less accessible nucleosomes and decreased transcription [48].

The role of acetylation on non-histone transcription factors is more varied. Acetylation may regulate protein subcellular localization, dimerization, protein–protein interaction, signal transduction, DNA binding and protein enzymatic activities. Acetylation may combine with other post-translational modifications, such as phosphorylation, methylation and ubiquitination to modulate protein activities [49]. Deacetylation has also been linked to the activation of non-histone enzymes such as acetyl-CoA synthetase [50].

Therefore, histone acetyltransferases and HDACs have multiple functions beyond histone modifications and may affect other proteins including transcription factors. Direct acetylation of transcription factors is emerging as a major determinant of regulating transcriptional activity much like phosphorylation in signal transduction. Different acetylases/deacetylases may associate with the same transcription factor, leading to different modifications in different cell populations or at different times.

Our recent studies have identified dynamic ensembles of the FOXP3 complex containing histone modification and chromatin-remodelling enzymes [39]. FOXP3 directly associates with histone acetyltransferase TIP60 *in vivo* [51] and *in vitro* (B. Li and M.I. Greene, unpublished results). FOXP3 is subject to acetylation that is promoted by TIP60 [51]. FOXP3-mediated transcriptional repression is dependent on recruitment of TIP60 by the N-terminal proline-rich domain of FOXP3 [51]. Acetylation may promote FOXP3 function since HDAC inhibitor treatment leads to increased FOXP3 levels and enhanced Treg function *in vitro* and *in vivo* [42,52].

21.5 Protein lysine deacetylation

Four classes of HDAC, including four in class I, six in class II, seven in class III and one in class IV, have been identified. Since HDACs can also deacetylate non-histone proteins, histone acetyltransferases and HDACs have been renamed lysine acetyltransferases (KATs) and lysine deacetylases, respecitvely, according to new nomenclature [10,53]. Class I lysine deacetylases – HDAC1, HDAC2, HDAC3 and HDAC8 – are broadly expressed in various types of cell and tissue. Class II lysine deacetylases – including HDAC4, HDAC5, HDAC6, HDAC7 and HDAC9 – and class IV lysine deacetylase – HDAC11 – are expressed in a tissue- or cell-specific manner. The

HDAC activity of class II lysine deacetylases is generally regulated by post-translational modification, especially phosphorylation, and is also regulated by their subcellular localization. Class III lysine deacetylases feature the sirtuins (which stands for silent mating type information regulation 2 homolog 1) of *Saccharomyces cerevisiae* – SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7 – are the homologues of yeast silent information regulator 2 (SIR2) protein, and require NAD as a co-enzyme [54]. The FOXP3 complex contains various class I and class II HDACs including HDAC1, HDAC7 and HDAC9 [51,55]. HDAC inhibitor treatment may enhance FOXP3 binding to chromatin (A. Samanta *et al.*, unpublished results) and promote murine *Foxp3* expression and Treg function *in vivo* [42].

21.6 HDAC inhibitors in autoimmune disease

In vivo administration of the HDAC inhibitor trichostatin A (TSA) ameliorated autoimmune lupus features in the MRL-*lpr/lpr* mouse strain, an effect that was attributed to affecting histone acetylation [16]. TSA's effects on histone acetylation also benefited experimental autoimmune encephalomyelitis [56]. TSA was believed to protect neurons by promoting antioxidants and counteracting pro-proliferative and apoptotic signals. TSA reduced $CD4^+$ T cell responses *in vitro* and *in vivo* through down-modulation of CD28 by an undefined mechanism involving histone modifications [57]. Another HDAC inhibitor, valproic acid, was also able to suppress cytokine production and limit autoimmune colitis in rodents through a modification of histone acetylation [58,59].

More recent studies have indicated that HDAC inhibitors can affect a variety of reactions including dampening alloreactivity and graft rejections by directly enhancing Treg function [42,52]. The effects of HDAC inhibitors on Treg function were assessed *in vivo* in three small-animal models [42]. First, $CD4^+CD25^-$ T cells were co-transferred with HDAC inhibitor- or DMSO-treated congenic Tregs into immunodeficient mice. By day 7, co-administration of wild-type Tregs with $CD4^+CD25^-$ T cells significantly decreased homeostatic T cell proliferation compared with transfer of $CD4^+CD25^-$ cells alone; transfer of TSA-treated Tregs also further decreased T cell proliferation. Second, in the dextran sulphate sodium (DSS)-induced CD4-dependent model of colitis, use of HDAC inhibitors increased $CD4^+FOXP3^+$ Tregs in lymphoid tissues and diminished disease severity. The effects were Treg-dependent, as prior CD25 monoclonal antibody therapy rendered mice unresponsive to TSA. Third, the effects of the HDAC inhibitor TSA on T cell-dependent alloresponses *in vivo* using responder fully MHC-mismatched cardiac and islet transplant models were studied. Cardiac allografts in control DMSO-treated mice were rejected similarly to untreated C57BL/6 mice, but TSA therapy for 14 days prolonged allograft survival. In addition, in studies now being completed, we found that valproic acid treatment decreased the incidence and severity of collagen-induced arthritis and acted therapeutically on the ongoing condition in mice (S.J. Saouaf and M.I. Greene, unpublished work).

21.7 Dietary butyrate promotes lysine acetylation by inhibiting deacetylases

Butyrate promotes histone lysine acetylation in a rapid and reversible way [60]. Dietary fibre can be processed by lactate-utilizing bacterial fermentation in the human intestine, which produces butyrate [61]. Riggs and colleagues showed that *n*-butyrate treatment could reversibly increase histone H4 acetylation in HeLa and leukaemic cells [60]. Candido *et al*. observed that sodium butyrate promotes the accumulation of acetylated histones by inhibiting deacetylation both *in vivo* and *in vitro* [62]. Hyperacetylation of histones after treatment with sodium butyrate is due to the inhibition of HDAC.

21.8 The HDAC inhibitor butyrate affects TGF-β signalling and increases Smad3 levels

Sodium butyrate may affect cell growth and phenotype by regulating the activity of transcription factors. Nguyen *et al*. have found that sodium butyrate could induce Smad3 and potentiate TGF-β signalling [63]. Increases of Smad3 mRNA and protein levels were apparent in a variety of non-transformed cell lines within 24 h of exposure to relatively low levels of sodium butyrate (5 mM). Moreover, sodium butyrate was also found to enhance TGF-β signals that led to Smad3 phosphorylation. Finally sodium butyrate and TGF-β were able to cooperatively reduce phenotypic features of cells such as anchorage-independent growth [63]. Acting in an autocrine and paracrine manner, TGF-β, as well as its downstream signalling pathway, plays a critical role in leucocyte biology [39]. TGF-β signalling regulates the development, differentiation, survival and proliferation of multiple cell lineages, including Tregs [64]. Some of the effects of TGF-β on Tregs have been mapped to the murine *Foxp3* enhancer [40].

21.9 HDAC inhibitors affect immune-cell proliferation and conversion of antigen triggered T cells into an unresponsive state

Butyrate has multiple biological effects in cultured cells by serving as an effective suppressor of cell proliferation and an inhibitor of cell-cycle progression, and by promoting cell differentiation in cancer cells [65]. These effects were also observed in immune cells [66]. Gilbert and Weigle found that *n*-butyrate blocked primary Th1 cells in the G_1 phase of the cell cycle and, in association with T cell receptor signalling, caused anergy rather than activation [66]. One mechanism by which butyrate blocks cell-cycle progression involves increased levels of cyclin-dependent kinase inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ in Th1 cells. p21 prevents cyclin-dependent kinase-mediated activation of retinoblastoma protein via phosphorylation, thus preventing cell-cycle entry to the S phase.

Gilbert and colleagues observed that butyrate species converted the phenotype of T cells which had encountered antigen into unresponsive cells [67]. While sodium butyrate has a very short half-life *in vivo* and its activity is thus limited, a number of modifications yield butyrate forms that have butyrate as breakdown species, thereby yielding longer *in vivo* activities. These modified forms include *n*-butyrate 2-(4-morpholinyl) ethyl butyrate hydrochloride (MEB) which has been studied *in vivo* and is active in immune modulation [67]. Populations of Th1 cells exposed to butyrate alone or to butyrate and IL-2 did not lose their ability to respond to a subsequent antigen challenge. Splenic T cells stimulated *in vitro* with an alloantigen in the presence of butyrate lost their ability to proliferate in response to the subsequent challenge with the initial alloantigen, but retained their ability to proliferate when stimulated with a third-party alloantigen. These data indicate that butyrate converts T cells, which have encountered antigen, into cells unable to react to antigen. Cells that have not encountered antigen are not directly converted into an unresponsive state.

21.10 Conclusions

Recently we identified FOXP3, the key transcription factor for Treg development and function, as an acetylated protein [51]. FOXP3 function in Tregs depends

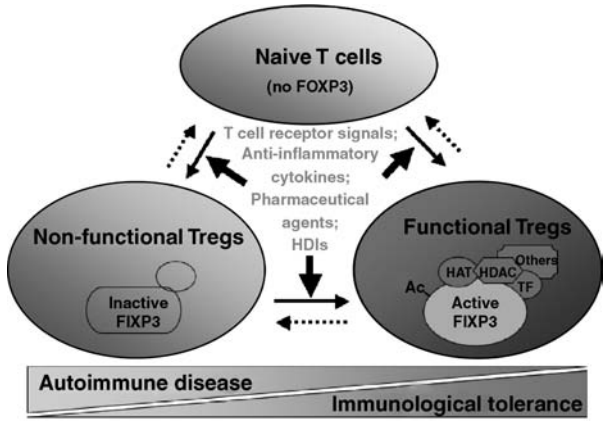

Figure 21.1 A working model of preventing autoimmune disease by inducing functional FOXP3$^+$ Tregs through deacetylase inhibition. T cell receptor signals, anti-inflammatory cytokines and pharmaceutical agents may modulate the epigenetic status of the *FOXP3* gene locus to induce FOXP3 expression, leading to the development and function of natural and inducible FOXP3$^+$ Tregs. However, functional FOXP3$^+$ Tregs may not only depend on FOXP3 expression alone, but also on post-translational modifications, such as acetylation (Ac) of FOXP3, as well as the complex ensemble of active FOXP3 with its binding partners, including transcription factors (TF), histone acetyltransferase (HAT) and HDAC. HDAC inhibitors (HDIs) promote FOXP3 expression, FOXP3 acetylation and the production of functional Tregs *in vitro* and *in vivo*. Consequently, functional FOXP3$^+$ Tregs control the immune system to prevent autoimmune disease.

on its dynamic complex ensemble with other enzymatic subunits. The N-terminal transcription-repression domain of FOXP3 recruits the histone acetyltransferase–HDAC complex, which is essential for its suppressive function [51]. Moreover, HDAC inhibitor treatment promotes the binding of FOXP3 to chromatin (A. Samanta, B. Li and M.I. Greene, unpublished results) and increases murine *Foxp3* gene expression, as well as the suppressive function of Tregs *in vivo* [42]. Our studies provide an important framework for molecular insights into how HDAC inhibitors regulate autoimmune disease by modulating the expression and post-translational modification of FOXP3, which leads to the enhanced Treg function (illustrated in Figure 21.1).

Since HDAC inhibitors have multiple molecular targets *in vivo* and demonstrate different binding affinities [68], we must keep in mind that they may mediate immunosuppression through multiple mechanisms. Moreover, histone deacetylation on select gene promoters may contribute to the transcriptional repression of pro-inflammatory genes in tolerant immune cells as well [69].

References

1. Goodnow, C.C. (2007) Multistep pathogenesis of autoimmune disease. *Cell* **130**, 25–35.
2. Sakaguchi, S., Yamaguchi, T., Nomura, T. *et al.* (2008) Regulatory T cells and immune tolerance. *Cell* **133**, 775–787.
3. Paust, S. and Cantor, H. (2005) Regulatory T cells and autoimmune disease. *Immunological Reviews* **204**, 195–207.
4. Roncarolo, M.G. and Battaglia, M. (2007) Regulatory T-cell immunotherapy for tolerance to self antigens and alloantigens in humans. *Nature Reviews Immunology* **7**, 585–598.
5. Feinberg, A.P. (2007) Phenotypic plasticity and the epigenetics of human disease. *Nature* **447**, 433–440.
6. Strickland, F.M. and Richardson, B.C. (2008) Epigenetics in human autoimmunity. Epigenetics in autoimmunity - DNA methylation in systemic lupus erythematosus and beyond. *Autoimmunity* **41**, 278–286.
7. Bird, A. (2007) Perceptions of epigenetics. *Nature* **447**, 396–398.
8. Goldberg, A.D., Allis, C.D. and Bernstein, E. (2007) Epigenetics: a landscape takes shape. *Cell* **128**, 635–638.
9. Yang, X.J. and Seto, E. (2007) HATs and HDACs: from structure, function and regulation to novel strategies for therapy and prevention. *Oncogene* **26**, 5310–5318.
10. Allis, C.D., Berger, S.L., Cote, J. *et al.* (2007) New nomenclature for chromatin-modifying enzymes. *Cell* **131**, 633–636.
11. Imhof, A., Yang, X.J., Ogryzko, V.V. *et al.* (1997) Acetylation of general transcription factors by histone acetyltransferases. *Current Biology* **7**, 689–692.
12. Tang, X., Gao, J.S., Guan, Y.J. *et al.* (2007) Acetylation-dependent signal transduction for type I interferon receptor. *Cell* **131**, 93–105.
13. Chung, Y.L., Lee, M.Y., Wang, A.J. *et al.* (2003) A therapeutic strategy uses histone deacetylase inhibitors to modulate the expression of genes involved in the pathogenesis of rheumatoid arthritis. *Molecular Therapy* **8**, 707–717.
14. Li, N., Zhao, D., Kirschbaum, M. *et al.* (2008) HDAC inhibitor reduces cytokine storm and facilitates induction of chimerism that reverses lupus in anti-CD3 conditioning regimen. *Proceedings of the National Academy of Sciences USA* **105**, 4796–4801.

15. Mishra, N., Brown, D.R., Olorenshaw, I.M. *et al.* (2001) Trichostatin A reverses skewed expression of CD154, interleukin-10, and interferon-gamma gene and protein expression in lupus T cells. *Proceedings of the National Academy of Sciences USA* **98**, 2628–2633.
16. Garcia, B.A., Busby, S.A., Shabanowitz, J. *et al.* (2005) Resetting the epigenetic histone code in the MRL-lpr/lpr mouse model of lupus by histone deacetylase inhibition. *Journal of Proteome Research* **4**, 2032–2042.
17. Mishra, N., Reilly, C.M., Brown, D.R. *et al.* (2003) Histone deacetylase inhibitors modulate renal disease in the MRL-lpr/lpr mouse. *Journal of Clinical Investigation* **111**, 539–552.
18. Camelo, S., Iglesias, A.H., Hwang, D. *et al.* (2005) Transcriptional therapy with the histone deacetylase inhibitor trichostatin A ameliorates experimental autoimmune encephalomyelitis. *Journal of Neuroimmunology* **164**, 10–21.
19. Miranda, T.B. and Jones, P.A. (2007) DNA methylation: the nuts and bolts of repression. *Journal of Cellular Physiology* **213**, 384–390.
20. Bernstein, B.E., Meissner, A. and Lander, E.S. (2007) The mammalian epigenome. *Cell* **128**, 669–681.
21. von Boehmer, H. (2005) Mechanisms of suppression by suppressor T cells. *Nature Immunology* **6**, 338–344.
22. Sakaguchi, S. and Powrie, F. (2007) Emerging challenges in regulatory T cell function and biology. *Science* **317**, 627–629.
23. Hori, S., Nomura, T. and Sakaguchi, S. (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057–1061.
24. Chen, W., Jin, W., Hardegen, N. *et al.* (2003) Conversion of peripheral CD4 + CD25- naive T cells to CD4 + CD25 + regulatory T cells by TGF-beta induction of transcription factor Foxp3. *Journal of Experimental Medicine* **198**, 1875–1886.
25. Zheng, S.G., Gray, J.D., Ohtsuka, K. *et al.* (2002) Generation ex vivo of TGF-beta-producing regulatory T cells from CD4 + CD25- precursors. *Journal of Immunology* **169**, 4183–4189.
26. Cobbold, S.P., Castejon, R., Adams, E. *et al.* (2004) Induction of foxP3 + regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants. *Journal of Immunology* **172**, 6003–6010.
27. Roncarolo, M.G., Gregori, S., Battaglia, M. *et al.* (2006) Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunological Reviews* **212**, 28–50.
28. Weiner, H.L. (2001) Oral tolerance: immune mechanisms and the generation of Th3-type TGF-beta-secreting regulatory cells. *Microbes and Infection* **3**, 947–954.
29. Lu, L., Werneck, M.B. and Cantor, H. (2006) The immunoregulatory effects of Qa-1. *Immunological Reviews* **212**, 51–59.
30. Sakaguchi, S. (2004) Naturally arising CD4 + regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annual Review of Immunology* **22**, 531–562.
31. Ziegler, S.F. (2006) FOXP3: of mice and men. *Annual Review of Immunology* **24**, 209–226.
32. Hill, J.A., Feuerer, M., Tash, K. *et al.* (2007) Foxp3 transcription-factor-dependent and -independent regulation of the regulatory T cell transcriptional signature. *Immunity* **27**, 786–800.
33. Hori, S. (2008) Rethinking the molecular definition of regulatory T cells. *European Journal of Immunology* **38**, 928–930.
34. Rudensky, A.Y. and Campbell, D.J. (2006) In vivo sites and cellular mechanisms of T reg cell-mediated suppression. *Journal of Experimental Medicine* **203**, 489–492.
35. Chen, G.Y., Chen, C., Wang, L. *et al.* (2008) Cutting edge: Broad expression of the FoxP3 locus in epithelial cells: a caution against early interpretation of fatal inflammatory diseases following in vivo depletion of FoxP3-expressing cells. *Journal of Immunology* **180**, 5163–5166.
36. Zuo, T., Wang, L., Morrison, C. *et al.* (2007) FOXP3 is an X-linked breast cancer suppressor gene and an important repressor of the HER-2/ErbB2 oncogene. *Cell* **129**, 1275–1286.

37. Hinz, S., Pagerols-Raluy, L., Oberg, H.H. *et al.* (2007) Foxp3 expression in pancreatic carcinoma cells as a novel mechanism of immune evasion in cancer. *Cancer Research* **67**, 8344–8350.
38. von Boehmer, H. and Nolting, J. (2008) What turns on Foxp3? *Nature Immunology* **9**, 121–122.
39. Li, B., Samanta, A., Song, X. *et al.* (2006) FOXP3 ensembles in T-cell regulation. *Immunological Reviews* **212**, 99–113.
40. Tone, Y., Furuuchi, K., Kojima, Y. *et al.* (2008) Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. *Nature Immunology* **9**, 194–202.
41. Kim, H.P. and Leonard, W.J. (2007) CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. *Journal of Experimental Medicine* **204**, 1543–1551.
42. Tao, R., de Zoeten, E.F., Ozkaynak, E. *et al.* (2007) Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nature Medicine* **13**, 1299–1307.
43. Zorn, E., Nelson, E.A., Mohseni, M. *et al.* (2006) IL-2 regulates FOXP3 expression in human CD4 + CD25 + regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo. *Blood* **108**, 1571–1579.
44. Marmorstein, R. (2004) Structural and chemical basis of histone acetylation. *Novartis Foundation Symposium* **259**, 78–98; discussion 98–101, 163–109.
45. Lee, H.J., Chun, M. and Kandror, K.V. (2001) Tip60 and HDAC7 interact with the endothelin receptor a and may be involved in downstream signaling. *Journal of Biological Chemistry* **276**, 16597–16600.
46. Dequiedt, F., Kasler, H., Fischle, W. *et al.* (2003) HDAC7, a thymus-specific class II histone deacetylase, regulates Nur77 transcription and TCR-mediated apoptosis. *Immunity* **18**, 687–698.
47. Roth, S.Y., Denu, J.M. and Allis, C.D. (2001) Histone acetyltransferases. *Annual Review of Biochemistry* **70**, 81–120.
48. Wolffe, A.P. (1996) Histone deacetylase: a regulator of transcription. *Science* **272**, 371–372.
49. Brooks, C.L. and Gu, W. (2003) Ubiquitination, phosphorylation and acetylation: the molecular basis for p53 regulation. *Current Opinion in Cell Biology* **15**, 164–171.
50. Starai, V.J., Celic, I., Cole, R.N. *et al.* (2002) Sir2-dependent activation of acetyl-CoA synthetase by deacetylation of active lysine. *Science* **298**, 2390–2392.
51. Li, B., Samanta, A., Song, X. *et al.* (2007) FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. *Proceedings of the National Academy of Sciences USA* **104**, 4571–4576.
52. Tao, R., de Zoeten, E.F., Ozkaynak, E. *et al.* (2007) Histone deacetylase inhibitors and transplantation. *Current Opinion in Immunology* **19**, 589–595.
53. Yang, X.J. and Seto, E. (2008) The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. *Nature Reviews Molecular Cell Biology* **9**, 206–218.
54. Denu, J.M. (2005) The Sir 2 family of protein deacetylases. *Current Opinion in Chemical Biology* **9**, 431–440.
55. Li, B., Saouaf, S.J., Samanta, A. *et al.* (2007) Biochemistry and therapeutic implications of mechanisms involved in FOXP3 activity in immune suppression. *Current Opinion in Immunology* **19**, 583–588.
56. Camelo, S., Iglesias, A., Hwang, D. *et al.* (2005) Transcriptional therapy with the histone deacetylase inhibitor trichostatin A ameliorates experimental autoimmune encephalomyelitis. *Journal of Neuroimmunology* **164**, 10–21.
57. Moreira, J., Scheipers, P. and Sorensen, P. (2003) The histone deacetylase inhibitor Trichostatin A modulates CD4 + T cell responses. *BMC Cancer* **3**, 30.
58. Glauben, R., Batra, A., Fedke, I. *et al.* (2006) Histone hyperacetylation is associated with amelioration of experimental colitis in mice. *Journal of Immunology* **176**, 5015–5022.
59. Gonzalez-Rey, E., Chorny, A., Del Moral, R.G. *et al.* (2006) Therapeutic effect of cortistatin on experimental arthritis by downregulating inflammatory and Th1 responses. *Annals of the Rheumatic Diseases* **66**, 582–588.

60. Riggs, M.G., Whittaker, R.G., Neumann, J.R. *et al.* (1977) n-Butyrate causes histone modification in HeLa and Friend erythroleukaemia cells. *Nature* **268**, 462–464.
61. Duncan, S.H., Holtrop, G., Lobley, G.E. *et al.* (2004) Contribution of acetate to butyrate formation by human faecal bacteria. *British Journal of Nutrition* **91**, 915–923.
62. Candido, E.P., Reeves, R. and Davie, J.R. (1978) Sodium butyrate inhibits histone deacetylation in cultured cells. *Cell* **14**, 105–113.
63. Nguyen, K.A., Cao, Y., Chen, J.R. *et al.* (2006) Dietary fiber enhances a tumor suppressor signaling pathway in the gut. *Annals of Surgery* **243**, 619–625; discussion 625–617.
64. Rubtsov, Y.P. and Rudensky, A.Y. (2007) TGFbeta signalling in control of T-cell-mediated self-reactivity. *Nature Reviews Immunology* **7**, 443–453.
65. Leder, A. and Leder, P. (1975) Butyric acid, a potent inducer of erythroid differentiation in cultured erythroleukemic cells. *Cell* **5**, 319–322.
66. Gilbert, K.M. and Weigle, W.O. (1993) Th1 cell anergy and blockade in G1a phase of the cell cycle. *Journal of Immunology* **151**, 1245–1254.
67. Gilbert, K.M., Wahid, R., Fecher, N.P. *et al.* (2000) Potential clinical use of butyric acid derivatives to induce antigen-specific T cell inactivation. *Journal of Pharmacology and Experimental Therapeutics* **294**, 1146–1153.
68. Dokmanovic, M., Clarke, C. and Marks, P.A. (2007) Histone deacetylase inhibitors: overview and perspectives. *Molecular Cancer Research* **5**, 981–989.
69. Foster, S.L., Hargreaves, D.C. and Medzhitov, R. (2007) Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature* **447**, 972–978.

Abstract

Abnormal epigenetic changes may alter the functional regulation of the immune system to self-antigens, leading to human autoimmune disorders. Regulatory T cells play an essential role in the control of autoimmunity. The forkhead family transcription factor FOXP3 regulates the development and function of natural regulatory T cells. Understanding the mechanisms underlying FOXP3 expression and function will lead to the ability to therapeutically modulate regulatory T cell function to treat autoimmune disease. Many factors, including cytokines and pharmaceutical agents, have been identified as the inducers of *FOXP3* gene expression. The induction of *FOXP3* expression also depends on the epigenetic status of the *FOXP3* gene promoter. Here we review recent work on treating autoimmune disease by histone deacetylase inhibitors, including new findings on modulating FOXP3 and regulatory T cells function through deacetylase inhibition.

Key words: autoimmune disease; regulatory T cell; epigenetics; FOXP3; acetyltransferase; deacetylase; deacetylation inhibitor; complex ensemble; butyrate.

22

Histone deacetylases and autoimmunity

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22.1 Introduction

Adequate functionality of the immune system is required for the protection of the organism against invaders and pathogens. However, inappropriate functioning leads to disease: loss of immune function increases the risk of infections and cancer, while overactivation of immune cells leads to systemic inflammation. If immune cells react with self-epitopes, autoimmune disorders may develop. Therefore, several barriers must prevent against autoimmunity, including the induction of central and peripheral tolerance to autoantigens during T lymphocyte development, regulatory $Foxp3^+$ T lymphocytes that inhibit $CD4^+$ and $CD8^+$ cell activation, tight regulation of lymphocyte activation and commitment and apoptosis after antigen encounter. These defending mechanisms are realized through the coordinated action of different regulatory pathways that finally converge to altered expression of genes responsible for immune response. An increasing amount of data supports the notion that epigenetic modification (particularly acetylation and deacetylation) of chromatin structure is of central importance in different levels of defense against autoimmunity. This review summarizes data collected so far.

22.2 Chromatin acetylation and deacetylation

The human genome is packaged in the nucleus into chromatin, a dynamic macromolecular complex made up of repetitive units, the nucleosomes [1]. The nucleosome core

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is composed of 146 or 147 base pairs (reports are contradictory) of DNA wrapped in 1.65 turns around the histone octamer formed by an H3–H4 tetramer and two H2A–H2B dimers.

Histones are small basic positively charged proteins rich in lysine and arginine. The four nucleosomic histones involve two domains: the C-terminal domain, which is located inside the nucleosome core, and the N-terminal domain. There are 14 contact points between histones and DNA. These multiple interactions make the nucleosome one of the most stable protein–DNA complexes under physiological conditions [2].

The nucleosome is not simply a static unit. It possesses dynamic properties that are tightly regulated by various protein complexes. Lysine residues on H3 and H4 histone tails extending out of the nucleosome are subject to a vast array of post-translational modifications, including acetylation. Histone acetylation results in a change of the net charge of nucleosomes. Neutralization of positive charge and an increase in hydrophobicity by histone acetylation reduce the affinity of histones to DNA template and greatly loosen inter- or intranucleosomal DNA–histone interactions. The resulting altered nucleosome structure facilitates the binding of transcription factors to nucleosomal DNA and enhances transcription. Higher-order chromatin structure may also be altered as a result of covalent modifications of histone subunits.

22.3 Histone deacetylases and histone acetyltransferases

Histone deacetylases (HDACs) and histone acetyltransferases (HATs) are two opposing groups of enzymes involved in chromatin remodelling by modifying the acetylation state of histones [3]. HATs are responsible for histone acetylation, while HDACs catalyse deacetylation by cleaving acetyl groups. In general, histone acetylation is associated with transcriptional activation, while deacetylation results in tightening of nucleosomal integrity, restriction of the access of transcription factors and suppression of transcription [4].

Currently, 17 human genes that encode proven or putative HDACs have been identified. Based on the analogy to Rpd3 and Hda1 yeast proteins, they are classified as class I HDACs (that include HDAC1, HDAC2, HDAC3 and HDAC8) and class II HDACs (that include HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 and HDAC10 human enzymes). Class I HDACs are present exclusively in the nucleus, while class II HDACs are shuttled between the cytoplasm and the nucleus. SIRT1, a human protein analogue to a yeast transcriptional repressor, is also categorized as a HDAC and belongs to a distinct family called sirtuins.

In addition to histones, HDACs can deacetylate non-histone proteins as well [5] (Figure 22.1). Some HDACs reside in the cytoplasm where they may exert their effects on a growing list of acetylated non-histone proteins. Both cytoplasmic and nuclear proteins can undergo reversible acetylation. Acetylation of a protein can have many different effects. First, it can affect protein stability. Since both acetylation and ubiquitylation

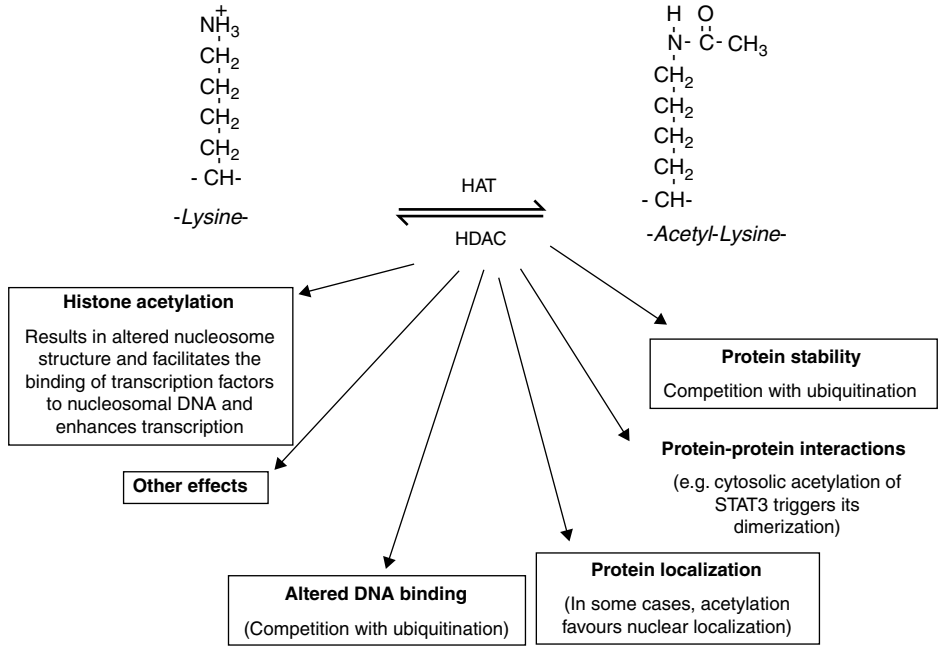

Figure 22.1 Effects of HDAC and HAT on histone and non-histone proteins.

target the same amino acid residue (lysine), competition between these two phenomena influences the stability of the substrate and therefore regulates its function indirectly. HDACs can decrease the half-life of several substrates by exposing the lysine residue to ubiquitylation. Second, acetylation affects protein–protein interactions. A characteristic example is the transcription factor signal transducer and activator of transcription 3 (STAT3), which is activated by cytokine signalling. Cytosolic acetylation of STAT3 triggers its dimerization and subsequent nuclear translocation. Acetylation of hypoxia-inducible factor 1 (HIF1) by the acetyltransferase ARD1 apparently leads to increased association with the von Hippel–Lindau (VHL) ubiquitylation complex and proteasome-mediated degradation, which has a regulatory role in the cellular response to changes in oxygen availability and angiogenesis [4]. Third, acetylation can have effects on protein localization. Reversible acetylation affects the subcellular localization of several proteins. In some cases the nuclear-localization signal contains acetylatable lysine residues that, when acetylated, favour nuclear retention. In the case of the multifunctional high-mobility group box 1 (HMGB1) protein, acetylation favours nuclear export and cytosolic accumulation before secretion during inflammatory and/or necrotic processes. Proteins involved in nuclear import can themselves be regulated by acetylation [4]. Fourth, acetylation can affect DNA binding. Thus, several transcription factors show increased DNA binding and subsequent transcriptional activity that correlate with the hyper-acetylation of histones in target chromatin. GATA3 and the p65 component of nuclear factor κB (NF-κB) are targets for acetylation and deacetylation, which thereby

modulate their transcriptional activity. Thus HDACs are also associated with inactive p65 and play a role in the regulation of NF-κB-mediated gene transcription without altering DNA binding. cAMP-response-element-binding protein (CREB)-binding protein (CBP) acetylates specific lysine residues on p65, increasing its binding to DNA and causing transcriptional activation. HDAC1 and HDAC2 are able to reverse this process. They can deacetylate acetylated NF-κB and promote its association with the inhibitor of NF-κB-α (IκB-α) within the nucleus to increase its export into the cytoplasm and, thus, terminate NF-κB activity. More surprisingly, in some cases acetylation impairs the binding of transcriptional activators to the DNA, indicating that HATs and HDACs might work in an orchestrated way to achieve the same cellular effect. Acetylation also impairs the catalytic and DNA-binding activities of enzymes involved in DNA metabolism and repair. Finally, acetylation can have other effects. In some cases, the function of HAT/HDAC substrates is influenced by acetylation at several levels. Similarly, the DNA-binding affinity of NF-κB (in its multiple forms) is regulated by HATs/HDACs, as is its transcriptional activation and its association with its regulator, IκB. Recently HDAC6 has been shown to deacetylate the cytoplasmic chaperone protein heat-shock protein 90 (HSP90). Hyperacetylation of this latter protein in HDAC6-deficient cells leads to the loss of chaperone activity [4].

Deacetylation inhibitors

The impact of HDACs on cell function is generally explored by the use of HDAC inhibitors (HDIs), a relatively wide range of compounds that derive from both natural and synthetic sources. With a few exceptions, they all can be divided into chemical classes including hydroxamic acid derivatives, carboxylates, benzamides, electrophilic ketones and cyclic peptides. The majority of HDIs are equally active against class I and class II HDACs. Widely used experimental substances for HDAC inhibition include trichostatin A (TSA), butyrate, suberoylanilide hydroxamic acid (SAHA), apicidin, valproic acid and hydroxamic acid-containing and benzamide-containing HDIs (for a detailed review see [1]).

HDIs induce up- as well as downregulation of a small subset of genes [6], suggesting that chromatin structure modulation by HDACs is a gene-specific event with variable transcriptional outcomes, and that only a few genes (approximately 2%) are regulated primarily through HDAC-dependent mechanisms.

HDAC inhibition of transcription is gene-specific in that expression of some genes is induced while that of others is repressed by HDIs. This suggests that HDACs can function not only as transcription repressors, as one would expect, but also as transcription activators depending on the specific promoter. The role of HDACs as repressors is understood as their recruitment to a promoter leads to histone deacetylation and chromatin condensation, a series of events which are favourable for transcriptional silencing [7]. However, it is still unclear how HDACs function as activators. It may be due to secondary effects. One possibility is that HDAC inhibition activates a transcription repressor which functions to silence the expression of targeted genes. It is

also possible that HDAC inhibition modifies the acetylation state of signalling proteins or transcription factors and affects their ability to activate specific promoters [8].

22.4 Histone acetylation, deacetylation and transcription factors in autoimmunity

HATs and HDACs form an integral part of nuclear co-activator and co-repressor complexes. An increasing number of data indicate their possible contribution to regulatory functions of transcription factors, including those responsible for the induction and maintenance of tolerance to self (Figure 22.2).

AIRE in central T cell tolerance

A central element in the development of T cell tolerance to self is the deletion of autoreactive clones in the thymus. Immature lymphocytes normally encounter only self-antigens at high concentrations and clones of lymphocytes whose receptors recognize these self-antigens with high affinity are deleted: this process is termed negative selection. A general assumption for negative selection is that thymocytes should encounter all self-antigens including those of extrathymic origin. Transcription factor autoimmune regulator (AIRE) is responsible for the expression of extrathymic proteins in the thymus (often termed the creation of an immunological shadow of

Cell type affected	HDAC target	Functional consequences
Thymocyte	AIRE	Altered development
Regulatory T cell	Foxp3	Altered functions
$CD4^+$ lymphocyte	CD154 CD86 MHC class II NF-κB STAT Tbet GATA3	Disturbed functions
Antigen presenting	IL-12, CD86	

Figure 22.2 Steps in the development of autoimmunity that are affected by HDAC. See text for definitions.

self) [9,10] and processing and/or presenting of self-antigens to maturing lymphocytes by thymic endothelial cells [11].

Fundamental roles of AIRE in the elimination of autoreactive T cells *in vivo* have been demonstrated by the use of a T cell receptor-transgenic mouse model [12]. In humans, the autoimmune syndrome autoimmune polyendocrinopathy/candidiasis/ectodermal dystrophy (APECED), characterized by mucocutaneous candidiasis, destruction of endocrine organs, including thyroid and parathyroid glands, and the production of antibodies to a variety of normal tissues, is the result of mutations of the AIRE gene [13,14].

Some observations suggest that HATs/HDACs may have an impact on both AIRE expression and the cellular effects of AIRE. For example, an increase of AIRE mRNA expression was seen in an experiment when cells were treated with TSA [15]. This indicates that, along with histone methylation, histone acetylation should participate in transcriptional control of AIRE. The possible involvement of HDACs in AIRE regulation could be explained by the documented presence of transcription factor-binding sites (nuclear factor-Y (NF-Y), activator protein 1 (AP-1) and Sp1) in AIRE promoter sequence. First, NF-Y, a common repressor of cell-cycle promoters, associates with HDAC1 [16], and is in dynamic interaction with HATs/HDACs [17]. The importance of HDACs has been established in the NF-Y-mediated repression of platelet-derived growth factor β (PDGF-β) receptors [18]. Second, HATs/HDACs are also of central importance in AP-1-driven gene regulation. For example, histone acetylation was found to be involved in fibroblast growth factor-mediated signalling leading to mesoderm induction [19]. Consistently, FR901228, an HDI, suppressed the expression of NF-κB and AP-1 in a leukaemia cell line [20]. Third, the transcription factor Sp1 is involved in gene expression in early development. Recent data suggest that the interaction of HDACs with Sp1 is critical for Sp1-mediated regulation of gene expression and/or that HDACs exert some of their regulatory functions through Sp1-binding sites [21].

No data regarding the direct importance of HDACs in AIRE-mediated regulation of gene expression are available. However, AIRE interacts with the common co-activator CBP [22]. CBP has intrinsic HAT activity, resulting in opening up of the chromatin structure, which allows binding of RNA polymerase II and initiation of gene transcription [23]. It is worth mentioning, however, that several other transcription factors interact with CBP, including NF-κB, AP-1 and STATs [4]. Conversely, a complex containing HDAC may repress CBP-induced gene expression.

Furthermore, the efficiency of antigen presentation may also be influenced by HDACs. Major histocompatibility complex (MHC) class II molecules play a fundamental part in the development of specific immune responses through their ability to present peptides to $CD4^+$ T lymphocytes and are critical for the specificity and efficiency of the immune response. Several reports have established the importance of epigenetic modulation as a positive regulator of MHC class II transcription [24]. Thus, HATs increase while HDACs repress MHC class II molecules via an interaction with master regulator class II transactivator (CIITA) [25]. Additionally, HATs/HDACs may have a CIITA-independent effect on MHC class II expression [26].

Foxp3 and regulatory T cells

Clonal deletion of autoreactive T cells is just the first line of defence against autoimmunity. In the periphery, $Foxp3^+CD4^+$ regulatory T cells (Tregs) are responsible for the suppression of improper immune responses. High numbers of Tregs have been linked to increased risk of cancer, while lack of this cell population (due to Foxp3 mutations) is the cause of IPEX, a rare and severe human autoimmune syndrome that is characterized by neonatal type I diabetes and autoimmune thyroiditis, recurrent infections, eczema, diarrhoea, chronic wasting and bleeding abnormalities [19].

Naturally, occurring Tregs emerge during thymic development and enter the periphery. In addition to emigration of thymus-derived Tregs to the periphery, conversion of $CD4^+CD25^-Foxp3^-$ cells into $CD4^+CD25^+Foxp3^+$ Tregs can also occur in the periphery. An increasing body of evidence suggests that histone acetylation and, particularly, HDACs determine the generation and repressive function of Foxp3 regulatory cells. A sophisticated series of experiments [27] in mice demonstrated the following. First, Tregs have a significantly higher HDAC activity than $CD4^+Foxp3^-$ cells. Primarily, they have nuclear an HDAC activity, but T cell receptor activation increases the expression of HDAC2 and HDAC3. Class II HDAC expression also differs modestly between $Foxp3^+$ and $Foxp3^-CD4^+$ cells. Second, HDAC9 is particularly important in regulating Treg-dependent suppression. Third, *in vivo*, HDIs induce expression of multiple Treg-associated genes without reversing Treg anergy. Finally, TSA increases the induction of Tregs either in the thymus or in the periphery. As a consequence of these alterations, HDI-treated Tregs are more effective at suppressing T cell proliferation than control Tregs. These results suggest that the use of specific inhibitors that target HDAC9 may interfere with the Foxp3–HDAC9 interaction and might have a beneficial effect in autoimmune disorders.

Acetylation state also is an important determinant of Foxp3-mediated gene repression mechanisms [28]. Foxp3 actively represses transcription through its association with HIV-1 Tat-interacting protein, 60 kDa (TIP60), a HAT enzyme, and HDAC7 and HDAC9 *in vivo*. The N-terminal 106–190-amino acid proline-rich region of Foxp3 has little similarity with other Foxp subfamily members and was identified as a critical region for Foxp3 forkhead domain-mediated transcriptional repression, dependent on its dynamic association with TIP60 and HDAC7. It was speculated that the association of Foxp3 with HAT/HDACs occurs to facilitate the preferential transcription of Foxp3-targeted genes and serves as a mechanism whereby cellular repression is established and regulated [29]. It has also been demonstrated that Foxp3 can even be acetylated in primary human Tregs and that acetylation can promote Foxp3-mediated transcriptional repression.

Apart from those identified in AIRE and Foxp3, there are no known mutations in transcription factors that induce autoimmune disease in humans [10]. However, other transcription factors that control lymphocyte differentiation and activity may also contribute to autoimmunity. HAT/HDAC function is also implicated in gene regulation by these transcription factors, such as STAT1 and -3, HIF-1, NF-κB and other intracellular proteins with immunoregulatory properties, such as nur77 and

glucocorticoid receptors. Their relationship with the acetylation status extends beyond the scope of this review.

22.5 Acetylation state and lymphocyte functions

Responses of effector $CD4^+$ cells are also modulated by HDACs. In peripheral blood mononuclear cells activation of lymphocytes increases HDAC mRNA levels, suggesting that HDACs are involved in cell-cycle progression and activation [30]. Indeed, HDACs have a profound impact on the activation of $CD4^+$ lymphocytes: they may contribute to increased CD4 density on the cell surface [31], to expression of a number of adhesion/co-stimulatory molecules and to expression of activation markers (CD154 [32], MHC class II [33] and CD86 [34]).

It has been revealed that HDAC inhibition in $CD4^+$ cells is associated with immunosuppression due to impaired activation of lymphocytes, growth arrest of T lymphocytes, inhibition of interleukin (IL)-2 mRNA levels or diminished levels of NF-κB compared to a control T cell population, indicating that HDIs affect NF-κB signalling. This latter mechanism is probably due to alteration of the acetylation state of p65 subunit of NF-κB: increased acetylation due to TSA reduced binding of p65 to NF-κB-containing DNA, facilitating its removal by IκBα and subsequent export to the cytoplasm [35]. As a net result of these effects, T cells undergo apoptosis.

In addition, T helper (Th) 1/Th2 ratios are profoundly influenced by HDACs. A balanced Th1 and Th2 response is critical to maintain immune homeostasis, and dysregulation of Th1 and Th2 activation leads to pathological immune responses. Lineage commitment in T cells is directed by the cytokine environment in which the T cell encounters antigens. IL-12 and IL-4 can strongly drive differentiation of Th1 and Th2 cells, respectively, via transcription factors such as STAT4 and STAT6. GATA3, expressed predominantly in Th2 cells, is critical for Th2 lineage development and may be the principal mediator of STAT6 function. Tbet is expressed predominantly in Th1 cells and influences interferon (IFN)-γ production and Th1 development. It is thought that the transcription factors GATA3 and Tbet are 'master regulators' of Th lineage determination [36].

Epigenetic events are important determinants of cytokine gene expression and lineage commitment of T lymphocytes. It has been shown that histones in the cytokine loci of naive T cells are unacetylated. Upon T cell receptor stimulation, the loci are rapidly and progressively acetylated on histones H3 and H4 and enable early transcription of cytokines, possibly contributing to the cytokine milieu in which T cell differentiation can proceed. The maintenance of acetylation depends on cytokine/STAT signalling. Tbet and GATA3 contribute to the polarized acetylated state. As transcription factor binding would highly favour an acetylated locus, maintained acetylation would provide locus and lineage specificity [37].

Importantly for immune memory, the histone-modification profile at a given locus can be inherited through mitosis. In cloned human T cells, the polarized acetylation pattern is retained through more than 20 cell divisions in the absence of polarizing

cytokines, suggesting that epigenetic modifications contribute to the maintenance of cytokine memory [38,39]. Quite recently it has been verified in clinical samples that endogenous HDAC activity plays a critical role in maintaining the level and balance of cytokine gene transcription in humans [40].

In addition to T lymphocytes, HDAC activities are required for innate immune cell control of Th1 effector cell function. Professional antigen-presenting cells, particularly macrophages and dendritic cells, are central players in the initiation of an inflammation cascade. They respond to diverse antigens, secrete cytokines and chemokines, modulate the expression of co-stimulatory molecules, and thereby instruct other innate and adaptive immune cells to mount appropriate responses [41,42]. Depending on the nature of the antigen (e.g. bacterial, viral or cell-associated) and the tissue microenvironment where they are activated, antigen-presenting cells develop into functionally different subsets. These distinct subsets can, in turn, promote specific immune processes, such as the activation of functionally distinct types of $CD4^+$ T cells. For example, addition of an HDI hydroxamic acid derivative to dendritic cells reduced their capacity to activate and attract Th1 effector cells, but preserved Th2 activation and migration signals [43].

22.6 HDACs and their inhibition in autoimmune disease

Although an increasing amount of data has been collected regarding HDACs in lymphocyte development and commitment, the direct role of HDAC in human autoimmune diseases is less well known. The majority of data regarding the possible relevance of HDACs in autoimmune disease have been obtained in HDI-treated experimental models of autoimmunity. One study reported human data on HDAC expression in type 1 diabetes mellitus (T1DM) [44]. Alterations in the balance of histone acetylation and deacetylation could affect many aspects of cellular function including cell growth, differentiation, cell death, cell–cell and cell–matrix interactions, and inflammatory responses. Thus, HDACs can represent therapeutic targets not only for cancer but also for other disorders, such as inflammatory diseases. Indeed, in recent years the anti-inflammatory effects of HDIs have attracted much attention. They appear to be quite effective in blocking inflammation and they may have an impact on the Th1/Th2 commitment of T lymphocytes (see above). Recent results have indicated that HDIs can reduce the cytokine and NO production that contributes to various inflammatory diseases [45]. Some of the most relevant observations in autoimmunity are summarized below.

Type 1 diabetes

Immune-mediated elimination of pancreatic β cells in T1DM involves release of cytotoxic cytokines such as IL-1β and IFN-γ, which induce β cell death *in vitro* by mechanisms that are both dependent and independent of NO. NF-κB is required for

the expression of the gene encoding inducible NO synthase and for that of pro-apoptotic genes in T1DM. Based on previous data showing the importance of epigenetic modulation in NF-κB signalling [46], a β cell line, INS-1, or intact rat islets, were precultured with HDIs (SAHA or TSA) in the absence or presence of IL-1β and IFN-γ. HDAC inhibition reduced cytokine-mediated decrease in insulin secretion and increase in inducible NO synthase levels, NO formation and apoptosis. IL-1β induced a biphasic phosphorylation of the inhibitor IκαBαl with the second peak being sensitive to HDAC inhibition. The authors concluded that HDAC inhibition prevents cytokine-induced β cell apoptosis and impaired β cell function associated with a downregulation of NF-κB transactivating activity.

In non-obese diabetic (NOD) mice, an animal model of T1DM, the administration of two structurally different HDIs (FR901228 and TSA) resulted in a profound inhibition of both $CD4^+$ and $CD8^+$ T-cell activation [47]. The HDIs inhibited IL-2 production from activated $CD4^+$ T cells and downregulated CD154 expression by a mechanism that is distinctly different from that of cyclosporin or FK-506. These preliminary *in vivo* studies suggested that FR901228 treatment can inhibit hyperglycaemia and reverse diabetes development in NOD mice.

However, a recent human observation does not fully support these results. In this report the human peripheral blood $CD4^+$ T cell genome-wide gene expression pattern and its relationship to the function of human $CD4^+$ T cells was studied [38]. Genes regulating the cell cycle were among the top-ranked genes affected. The cell-cycle analysis of $CD4^+$ T cells showed that T1DM $CD4^+$ cells were slow to enter into mitosis compared with $CD4^+$ cells of healthy controls or type 2 diabetic $CD4^+$ cells. This indicates a profound defect in the overall function of these cells. The results also suggested the involvement of some key regulatory elements. These include decreased expression of one key regulator, HDAC1, at the gene level and a similar tendency at the protein level. The authors concluded that decreased expression of HDAC in the $CD4^+$ cells in T1DM may play a key role in the defective function of these cells through altered epigenetic modification. The apparent contradiction between the result of this human study and those of *in vitro* and animal experiments cautions that observations made under laboratory conditions should not systematically be extrapolated into clinical therapy. Further studies should be conducted to resolve this issue.

Multiple sclerosis and experimental autoimmune encephalomyelitis

Multiple sclerosis is a Th1 cytokine-driven inflammatory, demyelinating and neurodegenerative disease of the central nervous system. Since multiple sclerosis is associated with transcriptional dysregulation [48] leading to Th1/Th2 skewness, an animal experiment was performed to test whether TSA would be clinically beneficial for experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis [49]. In this experiment TSA harnessed neuronal survival and anti-inflammatory pathways in experimental autoimmune encephalomyelitis, resulting in clinical amelioration during the chronic relapsing phase. It has been speculated that this HDI

provided neuroprotection by promoting antioxidant, anti-excitotoxicity, hormonal and growth responses, by counteracting pro-proliferative or pro-apoptotic E2F target genes and caspase-dependent and -independent apoptotic signals, and derepressing neuronal integrity traits.

Rheumatoid arthritis

The aberrant autoimmune response in rheumatoid arthritis is centred within the synovium membrane lining the joint. The first event in the pathogenesis of rheumatoid arthritis is probably the antigen-dependent activation of T cells, which subsequently leads to synovial infiltration of T and B lymphocytes and induction of pro-inflammatory mediators from macrophages and fibroblasts in the synovium. Dissection of the cytokine network in rheumatoid arthritis has localized several cytokines to the synovial tissue and led to the notion that hierarchical paracrine and autocrine pathways, with IL-1 and tumour necrosis factor α (TNF-α) assuming particular importance, can lead to the perpetuation of synovial inflammation and proliferation.

The therapeutic effects of topical phenylbutyrate and TSA were tested in rats with adjuvant arthritis, an animal model of rheumatoid arthritis [50]. In this study, it has been revealed that administration of these HDIs inhibits synovial fibroblast proliferation, reduces joint swelling, upregulates cell-cycle inhibitors $p16^{INK4}$ and $p21^{Cip1}$, suppresses pathological alterations in the joints, selectively induces cell-cycle inhibition in the synovium and pervasively suppresses TNF-α in affected tissues. In summary, this experiment has demonstrated that HDIs not only alleviated tissue inflammation, but also, if given earlier, prevented joint destruction in adjuvant arthritis. The effects of suppressing adjuvant arthritis correlated with the extent of histone hyperacetylation by the HDIs.

Another study investigated the possible effects of the HDI depsipeptide, also called FK228, on autoantibody-mediated arthritis. In that model, FK228 successfully inhibited joint swelling, synovial inflammation and subsequent bone and cartilage destruction in mice with autoantibody-mediated arthritis. It also affected two cell-cycle regulators, $p16^{INK4a}$ and $p21^{WAF1/Cip1}$, by inducing the expression of $p16^{INK4a}$ and upregulating that of $p21^{Cip1}$ [51].

More recently, the anti-rheumatic effects of SAHA and MS-275 were also assessed in both mouse and rat collagen-induced arthritis models [52]. SAHA exhibited moderate prophylactic efficacy. It attenuated paw swelling due to inflammation, decreased bone erosion in both mice and rats and reduced slightly rheumatoid arthritis-induced bone resorption in rats. However, SAHA could not inhibit the onset of arthritis. In contrast, MS-275 displayed dramatic anti-rheumatic effects. In prophylactic intervention, high doses of MS-275 prevented bone erosion and markedly delayed the onset of arthritis. At low doses, MS-275 strongly attenuated paw swelling, bone erosion and bone resorption associated with rheumatoid arthritis. Furthermore, the therapeutic efficacy of MS-275 was also documented. After the onset of arthritis, it could stop the disease progression and joint destruction. An anti-inflammatory effect of MS-275 was also confirmed

through its ability to decrease serum IL-6 and IL-1β levels in the collagen-induced arthritis mouse model. The anti-rheumatic activity of MS-275 was also confirmed through histological observations. No synovial hyperplasia, pannus formation, cartilage or bone destruction were observed in the high-dose prophylactic intervention in mice. The authors concluded that inhibition of HDAC may be important as a novel anti-rheumatic therapy.

Systemic lupus erythematosus

Systemic lupus erythematosus is an autoimmune disease characterized by heightened levels of cytokines produced by T cells, polyclonal B cell activation, dysregulated autoantibody production and renal inflammation. Interestingly, TSA and SAHA inhibited IL-6, IL-10, IL-12 and IFN-γ production by splenocytes of MRL-*lpr/lpr* mice, a model of systemic lupus erythematosus [53], as well as by human lupus T cells [54]. In splenocytes, HDIs induced histone H3 and H4 acetylation, but did not alter cell viability. Moreover, in glomerular mesangial cells stimulated with lipopolysaccharide and cytokines, TSA inhibited the production of TNF-α, IL-6, IL-12 and NO in a dose-dependent manner [55]. Finally, in MRL-*lpr/lpr* mice, subcutaneous injections of TSA ($0.5\,\mathrm{mg\,kg^{-1}}$) significantly reduced proteinuria, glomerulonephritis and splenomegaly, as well as the renal pathology index, without changing the circulating level of autoantibodies, immune-complex deposition and/or complement fixation in the glomerulus. However, autoantibody production may be also influenced by administration of HDIs. In the T347 cell line, which produces anti-DNA autoantibody, TSA markedly reduced the production of IgG2a anti-double-stranded DNA autoantibody in a dose-dependent manner [56]. Thus, HDIs could have therapeutic benefits in the treatment of systemic lupus erythematosus.

Scleroderma

Scleroderma is a systemic autoimmune disease of unknown aetiology and pathogenesis. It is characterized by progressive vasculopathy and widespread tissue fibrosis. Recent data suggest that an epigenetic mechanism may indeed lead to augmented collagen expression by scleroderma fibroblasts [57]. First, inhibitors of DNA methyltransferases and HDACs led to normalization of type I collagen expression levels in scleroderma fibroblasts, providing evidence of epigenetic influence on collagen gene expression. Second, the levels of factors involved in the maintenance of epigenetic mechanisms are clearly elevated in the nuclear extract from scleroderma fibroblasts, demonstrating the ability of the cells to sustain an epigenetic process. The signal transduction leading to the scleroderma fibrotic phenotype appears to converge to DNA methylation and histone deacetylation at the Ets gene family member *FLI1*.

Inflammatory bowel diseases

Inflammatory bowel diseases are a group of idiopathic inflammatory diseases of the colonic mucosa. The observation that butyrate and TSA inhibit IL-8 expression in colonic epithelial cells suggested that HDIs could be used for the effective treatment of ulcerative colitis through increased histone acetylation and reduced production of pro-inflammatory cytokines by the intestinal epithelium [58]. Additionally, butyrate inhibited dendritic cell maturation and IL-12 production [40], and reduced IL-2 transcription in T cells [39]. These results suggested that bacteria could escape the host defence in the gastrointestinal tract by producing high amounts of the HDI butyrate. Indeed, daily oral treatment of mice with $50\,mg\,kg^{-1}$ SAHA reduced the clinical and cytokine abnormalities in dextran sulphate sodium-induced colitis significantly [59]. Furthermore, a human clinical trial indicated that butyrate enema treatment (100 mM) for up to 8 weeks resulted in marked improvement or remission in ulcerative colitis [60]. The usefulness of local butyrate administration was also observed in ulcerative colitis that was refractory to conventional salicylate treatment [61]. Thus, HDIs hold great promise for treating human inflammatory bowel disease. However, at present there are few clinical data indicating that other HDIs could be used as effective anti-inflammatory drugs and appropriate studies are urgently required.

Psoriasis

It was postulated initially that keratinocyte hyperproliferation and the associated abnormal epithelial cell differentiation were the major culprits involved in the pathogenesis of psoriasis. More recently, the role of the immune system has become a major area of investigation, with keratinocyte hyperproliferation and epithelial cell differentiation being regarded as potential secondary events to immune dysfunction. Factors produced by T cells, such as IFN-γ, induce keratinocyte proliferation, blood-vessel formation and neutrophil infiltration in the skin. Furthermore, certain T lymphocyte subsets are present at high levels in the dermis of patients with psoriasis. Consequently, T cell-mediated inflammation is regarded as a key contributor to psoriasis [62].

In addition to the anti-inflammatory properties of HDIs discussed above, several studies have demonstrated that TSA and butyrate cause cell-cycle arrest in primary keratinocytes [26]. Growth arrest is accompanied by a decrease in RNA levels of the cyclin-dependent kinase 1 and an increased level of the differentiation marker TG1, suggesting that HDIs prompt differentiation in primary keratinocytes. The ability of HDIs to induce cell-cycle arrest associated with differentiation has been well documented in haematological malignancies and would clearly have an advantageous outcome in psoriasis [63]. HDI may also exert an anti-angiogenic potential and this property may also be of benefit for patients with psoriasis.

22.7 Conclusions

HDACs are implicated in different stages of mechanisms that culminate in the development of an autoimmune disease. They determine the accessibility of transcription factors to target gene sequences and, hence, play an important role in the regulation of gene expression. HAT/HDAC enzymes are integral parts of complexes implicated in regulation of *Foxp3* and *AIRE* as well. In addition, Th1/Th2 commitment of T lymphocytes also depends on the histone-acetylation state and, therefore, on HDAC function.

A number of HDIs have been developed and tested *in vitro* and in different types of model. Administration of these substances reversed Th1/Th2 skewness towards Th2 cells, inhibited proliferation of autoimmune T cell clones and conveyed powerful anti-inflammatory effects. The results suggest that HDIs could provide a safe and efficient means for immune modulation and may in the future join the therapeutic arsenal used to treat autoimmune disorders.

22.8 Acknowledgements

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References

1. Monneret, C. (2005) Histone deacetylase inhibitors. *European Journal of Medical Chemistry* **40**, 1–13.
2. Li, B., Carey, M. and Workman, J.L. (2007) The role of chromatin during transcription. *Cell* **128**, 707–719.
3. Huang, L. (2006) Targeting histone deacetylases for the treatment of cancer and inflammatory diseases. *Journal of Cellular Physiology* **209**, 611–616.
4. Pazin, M.J. and Kadonaga, J.T. (1997) What's up and down with histone deacetylation and transcription? *Cell* **89**, 325–328.
5. Minucci, S. and Pelicci, P.G. (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. *Nature Reviews Cancer* **6**, 38–51.
6. Van Lint, C., Emiliani, S. and Verdin, E. (1996) The expression of a small fraction of cellular genes is changed in response to histone hyperacetylation. *Gene Expression* **5**, 245–253.
7. Thiel, G., Lietz, M. and Hohl, M. (2004) How mammalian transcriptional repressors work. *European Journal of Biochemistry* **271**, 2855–2862.
8. Sun, H. and Taneja, R. (2000) Stra13 expression is associated with growth arrest and represses transcription through histone deacetylase (HDAC)-dependent and HDAC-independent mechanisms. *Proceedings of the National Academy of Sciences USA* **97**, 4058–4063.
9. Sigal, L.H. (2006) Basic science for the clinician 36: Protecting against autoimmunity: tolerance and Aire, the immunologic shadow, and other mechanisms of negative selection in the thymus. *Journal of Clinical Rheumatology* **12**, 44–46.

10. Sigal, L.H. (2006) Basic science for the clinician 37: Protecting against autoimmunity-tolerance: mechanisms of negative selection in the thymus. *Journal of Clinical Rheumatology* **12**, 99–101.
11. Kuroda, N., Mitani, T., Takeda, N. *et al.* (2005) Development of autoimmunity against transcriptionally unrepressed target antigen in the thymus of Aire-deficient mice. *Journal of Immunology* **174**, 1862–1870.
12. Liston, A., Lesage, J., Wilson, L. *et al.* (2003) Aire regulates negative selection of organ-specific T cells. *Nature Immunology* **4**, 350–354.
13. The Finnish-German, APECED Consortium (1997) An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains. *Nature Genetics* **17**, 399–403.
14. Nagamine, K., Peterson, P., Scott, H.S. *et al.* (1997) Positional cloning of the APECED gene. *Nature Genetics* **17**, 393–398.
15. Murumägi, A., Vähämurto, P. and Peterson, P. (2003) Characterization of regulatory elements and methylation pattern of the autoimmune regulator (AIRE) promoter. *Journal of Biological Chemistry* **278**, 1984–1990.
16. Peng, Y., Stewart, D., Li, W. *et al.* (2007) Irradiation modulates association of NF-Y with histone-modifying cofactors PCAF and HDAC. *Oncogene* **26**, 7576–7583.
17. Caretti, G., Salsi, V., Vecchi, C. *et al.* (2003) Dynamic recruitment of NF-Y and histone acetyltransferases on cell-cycle promoters. *Journal of Biological Chemistry* **278**, 30435–30440.
18. Uramoto, H., Wetterskog, D., Hackzell, A. *et al.* (2004) p73 competes with co-activators and recruits histone deacetylase to NF-Y in the repression of PDGF beta-receptor. *Journal of Cell Science* **117**, 5323–5331.
19. Bennett, C.L., Christie, J., Ramsdell, F. *et al.* (2001) The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nature Genetics* **27**, 20–21.
20. Mori, N., Matsuda, T., Tadano, M. *et al.* (2004) Apoptosis induced by the histone deacetylase inhibitor FR901228 in human T-cell leukemia virus type 1-infected T-cell lines and primary adult T-cell leukemia cells. *Journal of Virology* **78**, 4582–4590.
21. Kim, S., Kang, J.K., Kim, Y.K. *et al.* (2006) Histone deacetylase inhibitor apicidin induces cyclin E expression through Sp1 sites. *Biochemical and Biophysical Research Communications* **342**, 1168–1173.
22. Pitkänen, J., Doucas, V., Sternsdorf, T. *et al.* (2000) The autoimmune regulator protein has transcriptional transactivating properties and interacts with the common coactivator CREB-binding protein. *Journal of Biological Chemistry* **275**, 16802–16809.
23. Ito, K., Charron, C.E. and Adcock, I.M. (2007) Impact of protein acetylation in inflammatory lung diseases. *Pharmacology & Therapeutics* **116**, 249–265.
24. Spilianakis, C., Kretsovali, A., Agalioti, T. *et al.* (2003) CIITA regulates transcription onset viaSer5- phosphorylation of RNA Pol II. *EMBO Journal* **22**, 5125–5136.
25. Ting, J.P. and Trowsdale, J. (2002) Genetic control of MHC class II expression. *Cell* **109**, S21–S33.
26. Gialitakis, M., Kretsovali, A., Spilianakis, C. *et al.* (2006) Coordinated changes of histone modifications and HDAC mobilization regulate the induction of MHC class II genes by Trichostatin A. *Nucleic Acids Research* **34**, 765–772.
27. Tao, R., de Zoeten, E.F., Ozkaynak, E. *et al.* (2007) Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nature Medicine* **13**, 1299–1307.
28. Li B., Samanta A., Song X. *et al.* (2007) Greene FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. *Proceedings of the National Academy of Sciences USA* **104**, 4571–4576.
29. Masternak, K. and Reith, W. (2002) Promoter-specific functions of CIITA and the MHC class II enhanceosome in transcriptional activation. *EMBO Journal* **21**, 1379–1388.

30. Dangond, F., Hafler, D.A., Tong, J.K. *et al.* (1998) Differential display cloning of a novel human histone deacetylase (HDAC3) cDNA from PHA-activated immune cells. *Biochemical and Biophysical Research Communications* **242**, 648–652.
31. Kozłowska, A. and Jagodziński, P.P. (2006) Effect of Trichostatin A on CD4 surface density in peripheral blood T cells. *Folia Histochemica et Cytobiologica* **44**, 259–262.
32. Mishra, N., Brown, D.R., Olorenshaw, I.M. *et al.* (2001) Trichostatin A reverses skewed expression of CD154, interleukin-10, and interferon-γ gene and protein expression in lupus T cells. *Proceedings of the National Academy of Sciences USA* **98**, 2628–2633.
33. Magner, W.J. and Tomasi, T.B. (2000) Chromatin-immune connections: regulation of MHC and other genes. *Journal of Reproductive Immunology* **48**, 1–15.
34. Maeda, T., Towatari, M., Kosugi, H. *et al.* (2000) Up-regulation of costimulatory/adhesion molecules by histone deacetylase inhibitors in acute myeloid leukemia cells. *Blood* **96**, 3847–3856.
35. Moreira, J.M., Scheipers, P. and Sørensen, P. (2003) The histone deacetylase inhibitor Trichostatin A modulates $CD4^+$ T cell responses. *BMC Cancer* **3**, 30.
36. Avni, O., Lee, D., Macian, F. *et al.* (2002) T(H) cell differentiation is accompanied by dynamic changes in histone acetylation of cytokine genes. *Nature Immunology* **3**, 643–651.
37. Fields, P.E., Kim, S.T. and Flavell, R.A. (2002) Cutting edge: changes in histone acetylation at the IL-4 and IFN-gamma loci accompany Th1/Th2 differentiation. *Journal of Immunology* **169**, 647–650.
38. Messi, M., Giacchetto, I., Nagata, K. *et al.* (2003) Memory and flexibility of cytokine gene expression as separable properties of human T(H)1 and T(H)2 lymphocytes. *Nature Immunology* **4**, 78–86.
39. Smale, S.T. and Fisher, A.G. (2002) Chromatin structure and gene regulation in the immune system. *Annual Review of Immunology* **20**, 427–462.
40. Su, R.C., Becker, A.B., Kozyrskyj, A.L. *et al.* (2008) Epigenetic regulation of established human type 1 versus type 2 cytokine responses. *Journal of Allergy and Clinical Immunology* **121**, 57–63.
41. Banchereau, J., Briere, F., Caux, C. *et al.* (2000) Immunobiology of dendritic cells. *Annual Review of Immunology* **18**, 767–811.
42. Gordon, S. (1998) The role of the macrophage in immune regulation. *Research in Immunology* **149**, 685–688.
43. Brogdon, J.L., Xu, Y., Szabo, S.J. *et al.* (2007) Histone deacetylase activities are required for innate immune cell control of Th1 but not Th2 effector cell function. *Blood* **109**, 1123–1130.
44. Orban, T., Kis, J., Szereday, L. *et al.* (2007) Reduced $CD4^+$ T-cell-specific gene expression in human type 1 diabetes mellitus. *Journal of Autoimmunity* **28**, 177–187.
45. Säemann, M.D., Parolini, O., Böhmig, G.A. *et al.* (2002) Bacterial metabolite interference with maturation of human monocyte-derived dendritic cells. *Journal of Leukocyte Biology* **71**, 238–246.
46. Larsen, L., Tonnesen, M., Ronn, S.G. *et al.* (2007) Inhibition of histone deacetylases prevents cytokine-induced toxicity in beta cells. *Diabetologia* **50**, 779–789.
47. Skov, S., Rieneck, K., Bovin, L.F. *et al.* (2003) Histone deacetylase inhibitors: a new class of immunosuppressors targeting a novel signal pathway essential for CD154 expression. *Blood* **101**, 1430–1438.
48. Iglesias, A.H., Camelo, S., Hwang, D. *et al.* (2004) Microarray detection of E2F pathway activation and other targets in multiple sclerosis peripheral blood mononuclear cells. *Journal of Neuroimmunology* **150**, 163–177.
49. Camelo, S., Iglesias, A.H., Hwang, D. *et al.* (2005) Transcriptional therapy with the histone deacetylase inhibitor trichostatin A ameliorates experimental autoimmune encephalomyelitis. *Journal of Neuroimmunology* **164**, 10–21.
50. Chung, Y.L., Lee, M.Y., Wang, A.J. *et al.* (2003) A therapeutic strategy uses histone deacetylase inhibitors to modulate the expression of genes involved in the pathogenesis of rheumatoid arthritis. *Molecular Therapy* **8**, 707–717.

51. Nishida, K., Komiyama, T., Miyazawa, S. *et al.* (2004) Histone deacetylase inhibitor suppression of autoantibody-mediated arthritis in mice via regulation of p16INK4a and p21(WAF1/Cip1) expression. *Arthritis & Rheumatism* **50**, 3365–3376.
52. Lin, H.S., Hu, C.Y., Chan, H.Y. *et al.* (2007) Anti-rheumatic activities of histone deacetylase (HDAC) inhibitors in vivo in collagen-induced arthritis in rodents. *British Journal of Pharmacology* **150**, 862–872.
53. Mishra, N., Reilly, C.M., Brown, D.R. *et al.* (2003) Histone deacetylase inhibitors modulate renal disease in the MRL-lpr/lpr mouse. *Journal of Clinical Investigation* **111**, 539–552.
54. Mishra, N., Reilly, C.M., Brown, D.R. *et al.* (2001) Trichostatin A reverses skewed expression of CD154, interleukin-10, and interferon-gamma gene and protein expression in lupus T cells. *Proceedings of the National Academy of Sciences USA* **98**, 2628–2633.
55. Yu, Z., Zhang, W., Kone, B.C. *et al.* (2002) Histone deacetylases augment cytokine induction of the iNOS gene. *Journal of the American Society of Nephrology* **13**, 2009–2017.
56. Lu, Z.P., Ju, Z.L., Shi, G.Y. *et al.* (2005) Histone deacetylase inhibitor Trichostatin A reduces anti-DNA autoantibody production and represses IgH gene transcription. *Biochemical and Biophysical Research Communications* **330**, 204–209.
57. Wang, Y., Fan, P.S. and Kahaleh, B. (2006) Association between enhanced type i collagen expression and epigenetic repression of the FLI1 gene in scleroderma fibroblasts. *Arthritis & Rheumatism* **54**, 2271–2279.
58. Huang, N., Katz, J.P., Martin, D.R. *et al.* (1997) Inhibition of IL-8 gene expression in Caco-2 cells by compounds which induce histone hyperacetylation. *Cytokine* **9**, 27–36.
59. Leoni, F., Zaliani, A., Bertolini, G. *et al.* (2002) The antitumor histone deacetylase inhibitor suberoylanilide hydroxamic acid exhibits anti-inflammatory properties via suppression of cytokines. *Proceedings of the National Academy of Sciences USA* **99**, 2995–3000.
60. Lührs, H., Gerke, T., Müller, J.G. *et al.* (2002) Butyrate inhibits NF-kappaB activation in lamina propria macrophages of patients with ulcerative colitis. *Scandinavian Journal of Gastroenterology* **37**, 458–466.
61. Vernia, P., Annese, V., Bresci, G. *et al.* (2003) Topical butyrate improves efficacy of 5-ASA in refractory distal ulcerative colitis: results of a multicentre trial. *European Journal of Clinical Investigation* **33**, 244–248.
62. McLaughlin, F. and La Thangue, N.B. (2004) Histone deacetylase inhibitors in psoriasis therapy. *Current Drug Targets. Inflammation and Allergy* **3**, 213–219.
63. Saunders, N., Dicker, A., Popa, C. *et al.* (1999) Histone deacetylase inhibitors as potential anti-skin cancer agents. *Cancer Research* **59**, 399–404.

Abstract

Adequate functionality of the immune system is required for the protection of the organism against invaders and pathogens. If immune cells react with self-epitopes, different types of autoimmune disorder may develop. An increasing amount of data supports the notion that epigenetic modifications of chromatin, particularly acetylation and deacetylation, are of central importance at different levels of prevention of autoimmunity. This review summarizes data obtained in studies of the role of histone deacetylation in the development of autoimmune disorders, and suggests the possible roles of histone deacetylase inhibitors in their treatment.

Key words: histone; histone deacetylases; histone acetyltransferases; autoimmune disorders; histone deacetylase inhibitors

23

Histone deacetylase inhibitors as a therapeutic modality in multiple sclerosis

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23.1 Introduction

Multiple sclerosis (MS) is a common cause of neurological disability in young adults, and is a disease of the central nervous system. Put simply, MS damages the protective insulation (known as myelin) surrounding the nerves, resulting in reduced or lost bodily function. The severity of these effects differs per individual. Some people experience symptoms for a short period of time and afterward may remain symptom-free for periods or months or years, while others may experience a more steady progression of the disease. MS is commonly considered to be an autoimmune disease: one in which white blood cells target and attack the body's own cells. In MS this causes central nervous system inflammation, damage of the myelin and neurodegeneration.

In MS, areas of inflammation are known as lesions or plaques. Changes in the size, number and location of these lesions can determine both the type and severity of symptoms. Frequently, however, MS may be clinically silent, showing no increase in symptoms while continuing to show signs of disease activity within the central nervous system. Additionally, areas of thick scar tissue may eventually form along the areas of damaged myelin. The term multiple sclerosis originates from the discovery of these hardened plaques: *multiple* refers to many and *sclerosis* refers to scars (www.msassociation.org/).

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Mortality in patients is only slightly higher in MS patients when compared with that in the general population. However, it increases significantly for older patients and those with longer disease duration [1]. The cost of caring for this disease was recently estimated for Europe. The total annual cost of MS in Europe was estimated at €12.5 billion for the year 2005. Direct costs represented slightly more than half of the total cost (€6.0 billion), whereas informal care was estimated at €3.2 billion and indirect costs due to morbidity at €3.2 billion [2].

Despite intensive research, our understanding of MS has only recently resulted in new advances for the treatment of this disease. One area which has garnered significant insights concerns how the enzymes which regulate the aberrant gene transcription in MS affect the way DNA is packaged into chromatin in the cell.

The histone code is a well-established hypothesis describing the idea that specific patterns of post-translational modifications to histones act like a molecular code recognized and used by non-histone proteins to regulate specific chromatin functions. The pro-inflammatory environment is increasingly being recognized as a critical element for autoimmune degenerative diseases. In this chapter I will discuss the current knowledge supporting the notion that epigenetics play important regulatory roles in MS pathology, and the current development of histone deacetylase (HDAC) inhibitors (HDIs) as a potential therapeutic modality in the treatment this disease by modulating the pro-inflammatory environment.

23.2 Linking the histone code with MS

While a large body of evidence has emerged linking aberrant histone modifications to altered gene expression and prognosis in cancer [3,4], our knowledge surrounding these post-translational epigenetic modifications in MS is not as well established. In the following sections I will describe the current knowledge concerning various histone post-translational modifications, and the cellular machinery necessary for these modifications which have been implicated in the potential molecular pathogenesis of MS.

Histone citrullination

Methylation of arginine residues on histones occurs through the activities of protein arginine methyltransferases (PRMTs) [5], such as PRMT1, and PRMT4. Their removal is mediated via a process of deimination which converts the methyl-arginine to citrulline. The enzymes necessary for this conversion are called peptidylarginine deiminises (PADs). PAD4 was one of the first enzymes shown to regulate gene expression through its activities on histone arginine methylation [6]. PAD4 has now been shown to be over-expressed and activated in the central nervous system of MS patients [7]. Nuclear PAD4 expression resulted in enhanced expression of tumour necrosis factor α (TNF-α), increased histone citrullination, irreversible changes within

oligodendrocytes and demyelination [7]. A second member of this family, PAD2, has also been shown to localize to myelin, and to functionally deiminate arginine methylation in myelin basic protein. In agreement with PAD4, levels of PAD2 are also increased in the myelin of MS patients [8]. These results parallel the activities of histone acetyltransferases (HATs) and methyltransferases which can also modify additional proteins.

Histone acetylation

One of the most extensively studied histone modifications is histone acetylation. Clear links between aberrant histone acetylation and disease pathogenesis have been demonstrated, and extensive pharmaceutical efforts have brought forward agents targeting this modification to clinical trials resulting in US Food and Drug Administration approval for suberoylanilide hydroxamic acid (SAHA) or Vorinostat (Zolinza), for the treatment of cutaneous T cell lymphoma (CTCL) [9]. In the following sections I shall explore the current literature linking histone acetylation within neural settings, with a specific emphasis on how this modification plays important roles in MS pathogenesis.

23.3 Neuronal traits are modulated by HDAC transcription-factor complexes

A study carried out in 1975 examined the acetylation status of histones in neuronal fractions [10]. Most recently, it has been demonstrated that HDACs play important roles in neurone differentiation [11] and expression of neurone-specific genes [12], and indeed regulate diverse cues such as maternal grooming [13] and addiction [14].

One of the best-established mechanisms concerns the genes which are controlled by a silencer element (repressor element-1 (RE1)/neuronal restrictive silencer element (NRSE)). A specific protein repressor element silencing a transcription factor (NRSF, also known as REST) binds to this element and prevents the expression of these genes in non-neuronal cells [15,16]. REST is a critical protein in development as it is required for viability in knockout mice, transient expression of a dominant negative form of this protein causes precocious neuronal differentiation [17] and down-regulation of this gene is essential for proper neuronal differentiation [18]. A functional link to aberrant REST activity and selective loss of neurones has been shown in a study of Down's syndrome [19].

REST contains two distinct repressor domains, one located at the N-terminus and the other at the C-terminus of the protein [20,21]. Two distinct neuronal repressor complexes have now been isolated containing both REST and HDACs. The first of these complexes involves direct interactions with mSin3A/B at the N-terminus which then recruits HDACs to repress gene expression [22–25]. The C-terminal repression domain associates with a novel protein called CoREST [26]. This protein also interacts with HDACs through its SANT domain and has been shown to be essential for

repression [27], and in particular actively represses genes essential for neuronal phenotype [28]. The ATP-dependent remodelling complex SWI/SNF also plays a role in REST-mediated neuronal gene regulation as it has recently emerged that CoREST recruits several SWI/SNF members, indicating that active chromatin remodelling is an element in REST-mediated repression [29,30].

CoREST complexes have also been shown to contain lysine methyltransferases, and recently a LSD1–CoREST–CtBP co-repressor complex was shown to be required for late cell-lineage determination and differentiation during pituitary organogenesis [31].

Class II and class III HDACs play pivotal roles in the proliferation and differentiation of neurones [32–35]. SIRT1 has also been shown to protect primary cultures of cerebral granule neurones from FOXO-induced cell death [36], while inactivation of an MEF2D–HDAC5 complex by depolarization-mediated calcium influx protects cerebellar granule neurone survival [37].

23.4 Motor neurone genes modulated by HDACs

In addition to RE1-/NRSE-directed repression of specific genes, motor neurones have been shown to express unique combinations of LIM-type homeodomain factors to define motor-column identity by regulating particular gene-expression profiles [38]. LIM homeodomain transcription factors have been shown to be regulated through a complex containing HDACs [39], and as such motor neurone pathfinding may be guided through an HDAC-directed process.

23.5 The transcription factor E2F1, HDACs and neuronal survival mechanisms

An essential feature for neuronal survival has also been linked to constitutive repression of E2F1 transcriptional activity through HDAC proteins [40]. Elevated levels of E2F1 lead to neuronal apoptosis [41,42] and enhanced immune cell proliferation [43], factors that could be deleterious in MS. Using microarray analysis enhanced E2F pathway transcription was observed in peripheral blood mononuclear cells from patients with MS [44]. Subsequently, we demonstrated that HDIs reduce levels of E2F class I proteins *in vivo* [45], data that have been recapitulated by others *in vitro* [46,47]. These observations may therefore help to explain why HDIs block immune-cell proliferation [48] and enhance neuronal survival [49]. Valproic acid is commonly used as a mood stabilizer and anticonvulsant, but has also been identified as an HDI [50,51]. Treatment of cultured rat neurones with valproic acid prevents spontaneous apoptosis, indicating a role for HDAC inhibition in mediating the neuroprotective action of this drug [52]. In the following sections we will discuss the protective role of HDIs in the neuronal setting in more detail.

23.6 HDACs play important roles in stem cell neuronal differentiation

HDACs have also been shown to play important roles in neuronal stem cell differentiation. Using dominant negative stem cell lines expressing mutant, FLAG-tagged HDACs with reduced enzymic activity, Howard and colleagues found that mutant HDAC1 reduced differentiation to neurones by 50% [53]. The importance of HDACs in neuronal differentiation has also been demonstrated using HDIs. In an *in vitro* study on the effects of the HDI trichostatin A (TSA) on the differentiation pattern of embryonic mouse neuronal stem cells during culture in a minimal, serum-free medium, it was found that under these conditions TSA treatment increased neuronal differentiation of the neuronal stem cells and decreased astrocyte differentiation [54].

In lineage-committed oligodendrocyte precursor cells inhibition of HDAC activity acted as a priming event in the induction of developmental plasticity [55]. A similar study examining the ability of oligodendrocyte progenitors to acquire the identity of myelin-expressing cells or choose alternative fates found that the activity of HDACs was critical to these processes [56]. Emphasizing this finding, the transcription factor Yin Yang 1 (YY1) was shown to be a critical regulator of oligodendrocyte progenitor differentiation, acting as a lineage-specific repressor of transcriptional inhibitors of myelin gene expression (Tcf4 and Id4), through the recruitment of HDAC1 to their promoters during oligodendrocyte differentiation [57]. These studies underline the importance of HDACs in neuronal differentiation.

A direct role for HDACs in the regulation of neural stem cell proliferation has been shown where the orphan nuclear receptor TLX, a critical regulator of stem cell proliferation, was found to associate with HDAC3 and HDAC5. Inhibition of HDAC activity or knockdown of HDAC expression led to marked induction of TLX target gene expression and dramatically reduced neural stem cell proliferation [58].

REST is also critically involved with neural stem cell differentiation. Its activation is sufficient to cause neuronal differentiation [59]. REST complexes are able to both silence and repress neuronal genes in embryonic neural stem cells through the creation of chromatin environments that contain both repressive and active local epigenetic signatures [28,60,61].

23.7 HDIs lead to acetylation of the Sp1 transcription factor

The activity of the transcription factor Sp1 has been shown to be regulated by acetylation, while its gene expression is also intimately regulated via HDACs [62]. Acetylation of Sp1 is also seen in response to oxidative stress in neuronal cortical cell cultures [49]. Strikingly, HDIs can protect these neuronal cortical cells from oxidative stress-induced cell death, suggesting that acetylation of Sp1 in response to oxidative stress is a compensatory anti-oxidant response.

23.8 Immune-system effects of HDIs

T cells are classified by the cytokine profiles generated by each group upon activation [63,64], and within MS, pro-inflammatory (T helper (Th) 1 phenotype) $CD4^+$ T cells play important roles in pathogenesis [65]. Studies on Th1 cells have shown the importance of histone acetylation in Th1 cell-specific gene expression [66], and in the regulation of innate immune-cell control of Th1 cell function [67]. In the following sections I will describe the potential for targeting histone acetylation in the setting of MS.

$CD4^+$ T cells are maintained in peripheral lymphoid organs. Their levels are critical to adaptive immunity, and are maintained by a homeostatic balance between production and elimination. Disturbances in the elimination of $CD4^+$ T cells can result in autoimmunity. A recent report has shown that β-arrestin 1 (an adaptor protein), positively regulated the survival of $CD4^+$ T cells by enhancing the expression of the proto-oncogene *Bcl2*. This was shown to occur by β-arrestin regulation of the *Bcl2* promoter through histone H4 acetylation, and in a mouse model of MS, experimental autoimmune encephalomyelitis (EAE), over-expression of β-arrestin-1 led to increased susceptibility to EAE, while mice engineered to over-express β-arrestin-1 were much more resistant [68]. Memory $CD4^+$ T cell survival has also been shown to require the activity of the polycomb group (PcG) gene *Bmi1*, where it recruits activities to alter histone acetylation, histone methylation and DNA CpG methylation at the pro-apoptotic *Noxa* gene. In a memory Th2-dependent airway inflammation model, knockout of *Bmi1* resulted in significant attenuation of inflammation [69]. As Bmi1 would appear to recruit HDACs to regulate expression of *Noxa*, therapeutic targeting of HDACs may be a potential strategy for targeting memory $CD4^+$ cells in MS.

Interleukin (IL)-10 is a potent anti-inflammatory cytokine produced by T cells and activated macrophages, and administration of exogenous IL-10 in the central nervous system ameliorates EAE progression [70,71]. CD40 is an important co-stimulatory molecule between T cells and antigen-presenting cells, and is required for the adaptive immune response. Aberrant expression of CD40 is associated with MS [72]. In a series of experiments, Etty Benveniste and colleagues have shown that lipopolysaccharide induces CD40 expression in macrophages and microglia cells via induction of acetylation and phosphorylation on histones H3 and H4 through recruitment of the HATs cAMP-response-element-binding protein (CREB)-binding protein (CBP) and p300. This activation of CD40 could be ameliorated by treatments with IL-10 [73,74].

Th1 cells have been shown to be pathogenic in the EAE animal model of MS through increased production of interferon (IFN)-γ, IL-2 and TNF-α [75]. In antigen-presenting cells, IFN-γ induces the secretion of IL-12, a cytokine that potently stimulates differentiation of naive (Th0) cells into Th1 cells [76,77]. Murine models lacking the IL-12p40 gene are resistant to EAE while neutralizing antibodies to IL-12 inhibit the *in vivo* development of EAE [76,77]. Regulation of IL-12 gene expression has been shown to require the activities of HATs and HDACs [78], and the

potential use of HDIs in limiting IL-12 effects *in vitro* has been shown where sodium butyrate inhibits both T cell proliferation and expression of IL-12 and IFN-γ in human immune cells [48,79,80].

The pro-inflammatory cytokine IL-2 is also secreted by Th1 cells [81]. HDIs have been shown to both inhibit the expression of IL-2 [82,83], and alter IL-2 mediated gene expression [84]. Two mechanisms by which HDIs affect this activity have been identified. In the first, they disrupt a HDAC4–nuclear receptor co-repressor 2 (NCOR2) complex critically important for IL-2 promoter activation [85]. In the second identified mechanism, HDIs increased expression of the *Foxp3* gene, and concommitantly increased acetylation of the Foxp3 protein itself. This was found to enhance Foxp3 binding to the IL-2 promoter resulting in suppression of its transcription [86]. Indeed, a complex containing both HATs (Tip60) and HDACs (HDAC7 and HDAC9) was found to be responsible for the regulation of IL-2 in T cells [87]. We also have shown down-regulation of the IL-2 receptor by TSA [45], underlining the importance of HDACs in the regulation of the IL-2 pathway at several levels, and demonstrating the potential therapeutic benefits of HDIs in targeting this pathway.

Additional evidence for the potential use of HDIs in blocking $CD4^+$ T cells comes from early experiments which showed that sodium butyrate could induce antigen-specific unresponsiveness in $CD4^+$ T cells *in vivo*. Previous stimulation by antigen was found to be necessary for this effect as cells treated with sodium butyrate alone were unaffected [88]. The induction of antigen-specific T cell inactivation demonstrated that the butyric acid derivatives sequestered essentially all activated Th1 cells into the G_1 phase of the cell cycle, suggesting that HDIs have the potential to induce anergy in already activated $CD4^+$ T cells [89]. A comparative study using various well-established HDIs confirmed that all tested drugs were able to induce antigen-specific immune anergy in cloned and naïve murine $CD4^+$ T cells [90].

Th2 cells, a different type of $CD4^+$ T helper cells have anti-inflammatory, pro-humoral and protective roles in EAE, and produce various cytokines including IL-4, IL5, IL-10 or IL-13. Chromatin remodelling has been shown to be critical to the regulation of Th2-specific genes [91]. HDIs have been shown to increase the expression of IL-10 [80]. Regulation of major histocompatibility complex (MHC) class II gene expression is intimately linked to both HATs and HDACs. The transactivator CIITA is a critical component in MHC II gene expression and can function to regulate these genes at two levels: (1) it can be recruited to these gene promoters as part of an enhanceosome complex that includes the HATs p300 and CBP [92–94] and (b) CIITA itself has an intrinsic HAT activity [95]. Detailed analyses of these mechanisms have revealed that both the enhanceosome and CIITA make variable, promoter-dependent contributions to histone acetylation and transcription apparatus recruitment. As such, CIITA is generally implicated at multiple levels of MHC II gene activation, whereas the enhanceosome contributes in a CIITA-independent manner only at certain promoters [96]. The formation and function of the CIITA MHC II enhanceosome complex has been shown to be disrupted by a HDAC1–mSin3A complex [97].

Another positive regulator of MHC II genes is the RFXANK protein. This protein has been shown to associate in a complex with the class II HDACs HDAC4 and HDAC5, where they repress MHC II promoter activation [98,99]. The transcriptional repressor Blimp-1 (PRDI-BF1) has been shown to directly regulate the expression of CIITA [100,101]. This protein has been shown to associate with HDACs both indirectly through the recruitment of the Groucho family of co-repressors [102,103], and by directly recruiting HDAC1 or HDAC2 [104]. The expression of the class II histocompatibility locus antigen direct repeat (HLA-DR) genes has been shown to be repressed through the action of HDAC1 [105], and also by HDAC4 and HDAC5 [98]. HDIs have been shown to up-regulate MHC II gene expression [106,107]. This raises the issue that HDIs may appear to enhance the risk of disease progression in MS rather than abrogate it, as they may elevate antigen presentation by MHC to the T cell receptor, resulting in activation rather than abrogation of T cell activation.

Nevertheless, besides this first stimulatory event provided by MHC presentation of antigen, additional co-stimulatory signals are required for T cell activation. The binding of such co-stimulatory molecules on antigen-presenting cells on the T cell surface are required for T cells to undergo activation, clonal expansion and cytotoxicity. One such pathway consists of the binding of CD28 on T cells with B7.1 or B7.2 co-stimulatory molecules on the antigen-presenting cell. In a study of MS plaques compared against inflammatory stroke lesions from the same brain, it was found that while B7.2 expression was observed in both, expression of B7.1 was restricted to just the MS plaques [108,109]. Data suggesting that this would be a critical element found enhanced expression of B7.1 on B cells isolated from the peripheral blood and spinal fluid of MS patients compared to controls, while B7.2 levels were similar [110]. As such, a reduction in the expression of B7.1 might help ameliorate the pathogenesis of MS. In this regard, HDIs have been shown to prevent IFN-γ-induced up-regulation of B7.1 expression while enhancing constitutive as well as cytokine-induced expression of B7.2 [111]. This may have beneficial effects in MS, as B7.2 is suggested to have a pro-Th2 function.

Perivascular infiltration of inflammatory cells into the central nervous system is a hallmark of MS and requires both the adhesion and transmigration of these cells through the blood–brain barrier [112]. Matrix metalloproteinases (MMPs) may be critical to this transmigration process, allowing the inflammatory cells access to the central nervous system [113]. The majority of macrophages found in active and necrotic lesions of MS patients stain positive for MMPs 1, 2, 3 and 9, while a small number of astrocytes were also found to be MMP-2, -3 and -9-positive in acute and chronic MS lesions [114]. HDIs have been shown to have important effects on MMP expression. One early report indicated that treating epithelial cells with sodium butyrate led to a slight induction of MMP-9 [115]. However, other studies using the HDI apicidin or romidepsin found either no effect on MMP-9 expression, or significant down-regulation, whereas MMP-2 was found to be specifically down-regulated by both inhibitors [116,117], and indeed a separate study using sodium butyrate in rat colon cancer cells, found down-regulation of both MMP-2 and MMP-9 in these cells [118].

Further evidence supporting the potential of HDIs to regulate MMPs was demonstrated when a key negative regulator of matrix metalloproteinases (RECK) was found to be up-regulated following treatment with TSA, which inhibited MMP-2 activation in the cell line used [119]. As a caveat, butyrate has been shown to enhance the expression of stromelysin-1 (MMP-3) in mesenchymal cells, but only after the expression of this MMP had been induced by cytokines. By itself, butyrate was unable to stimulate stromelysin expression [120]. However, a recent study on the effects of TSA on joint inflammation and cartilage degeneration in a collagen antibody-induced arthritis mouse model found significant down-regulation of MMP-3 in the TSA-treated group, and TSA was also found to suppress IL-1β and TNF-α stimulated up-regulation of MMP-3 [121].

Regulation of MMP activity occurs via tissue inhibitors of metalloproteinases (TIMPs), whose up-regulation has clearly been observed in cells treated with various HDIs [122–127]. Treating MS patients with HDIs may therefore prevent the break-down of the blood–brain barrier and thus effectively block entry of inflammatory cells into the central nervous system.

23.9 HDACs and pro-inflammatory and stress-related pathways in immune settings

Inflammation is a critical component which is increasingly being associated with cancer [128,129], diabetes [130] and neurodegenerative disease [131]. Endoplasmic reticulum (ER) stress is another cellular event which has also been implicated as a critical component in cancer [132], diabetes [133], and neurodegeneration [134,135]. HDACs have been shown to be critical regulators of many important cellular processes, and evidence is emerging linking these enzymes in the regulation of inflammation and ER stress responses. Over the following sections of this chapter I shall link HDACs to both of these important pathways in the context of endocrine-related cancer, and immune and metabolic disorders. Subsequently, we shall discuss how targeting the HDACs may be important clinically, as they may be able to consequently alleviate symptoms of inflammation and ER stress in these conditions.

23.10 HATs, HDACs and the NF-κB pathway

Critical regulators of pro-inflammatory cascades identified to date include histone-modifying enzymes such as HDACs. The activation of nuclear factor B (NF-κB) is a key mediator of inflammatory cascades, and HDACs have been demonstrated to play important roles in regulating this activation process as shown in Figure 23.1. NF-κB typically consists of a heterodimeric protein comprised of a p50 and a RelA/p65 subunit, and initial studies identified the HATs p300 and CBP as key co-activators in regulating NF-κB-driven gene expression [136–138]. Another HAT, SRC-1, was found to also potentiate NF-κB transactivation through interactions with the other subunit p50 [139]. Following the identification of these interactions, it was subsequently shown

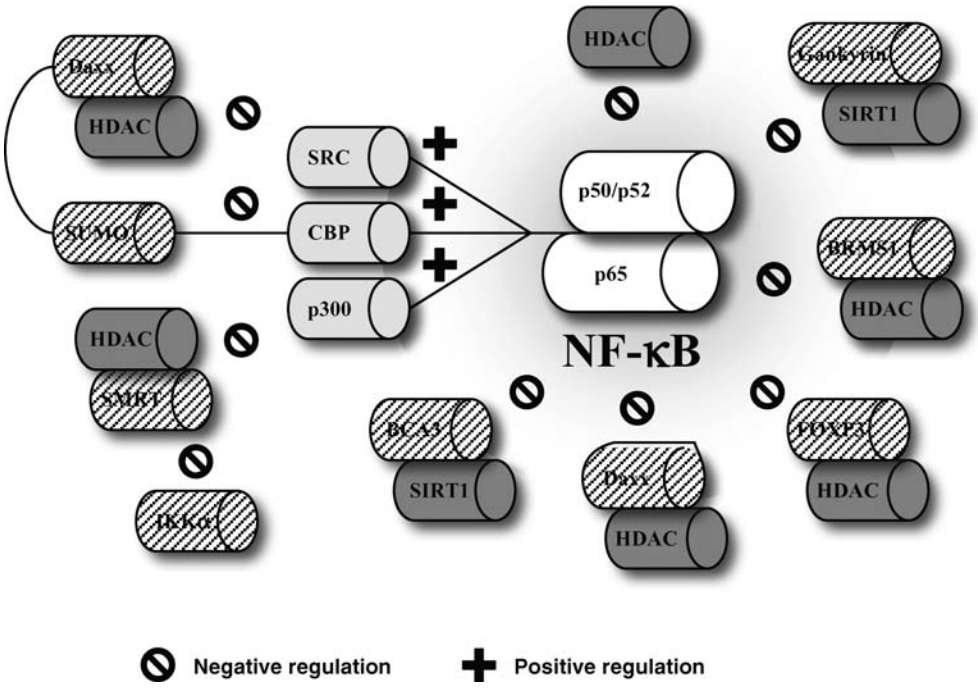

Figure 23.1 Diagramatic representation of the interplay between HATs and HDACs in the regulation of NF-κB. HATs are represented by light grey, HDACs are represented by dark grey and other interacting proteins are represented by hatching. BCA3, breast cancer-associated gene 3 (or protein kinase A-interacting protein 1); BRMS1, breast cancer metastasis suppressor 1; SMRT, silencing mediator for retinoid and thyroid hormone receptors (or nuclear receptor co-repressor 2, NCOR2).

that the RelA/p65 subunit could also associate with HDAC1 and HDAC2 to repress expression of NF-κB-regulated genes as well as to control the induced level of their expression [140]. It has since been shown that the HDAC SIRT1 regulates NF-κB transactivation by physically interacting with the RelA/p65 subunit of NF-κB and inhibiting transcription by deacetylating a critical lysine at position 310 [141]. One of the critical regulators of NF-κB activation is IκB kinase α (IKKα), where NF-κB transcription requires IKKα to phosphorylate silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) which stimulates the exchange of co-repressor for co-activator complexes. In the initial stage of NF-κB activation, following this phosphorylation event HDAC3 is displaced, allowing p300 to acetylate RelA/p65 [142,143].

Daxx is another protein which has been shown to regulate NF-κB activation by binding to a region which includes the major sites of acetylation mediated by p300/CBP [144]. However, it must be noted that Daxx has also been shown to directly associate with HDAC2 [145], and so may represent a mechanism by which HATs and HDACs compete for critical lysines on NF-κB subunits. In this regard, small ubiquitin-like

modifier (SUMO) modification of CBP negatively modulates its transcriptional activity by recruiting a Daxx complex which contains HDAC2 [146].

Pro-inflammatory genes associated with NF-κB include IL-6 and IL-8 [147–149]. NF-κB has also been shown to utilize the HAT activity of CBP/p300 to stimulate the transcription of these genes [150].

The forkhead box protein P3 (FOXP3) is a master cell lineage modulator in $CD4^+CD25^+$ natural regulatory T cell (Treg) development, and has also been shown to associate with HDACs 7 and 9 [87]. As FOXP3 has also been shown to interact with NF-κB to repress cytokine gene expression and effector functions of Th cells [151], it may be that the repression is mediated via HDAC activity. Evidence supporting this notion comes from a recent study where Foxp3 was shown to induce the silencing of IL-4 in primary Th2 cells via inhibition of nuclear translocation of NF-κB. This resulted in a decrease of *in vivo* binding of NF-κB to the IL-4 promoter, which was coupled with decreased levels of acetylated histone H3 at the IL-4 promoter [152].

Direct evidence for the role of NF-κB in MS has come from both experimental models of MS and from patient studies. One of the first indications of the importance of this pathway in MS pathogenesis came from a candidate gene study approach which identified both MS predisposing and protective alleles within NF-κB inhibitor genes (NF-κBIL1, predisposing; NF-κBIA, protective) [153]. However, in a study of peripheral blood mononuclear cells from MS patients, no abnormalities in NF-κB activity were observed between patients and controls [154].

It is well established that aberrant expression of CD40 (a member of the TNF receptor superfamily) is associated with MS [72]. In macrophages and microglia it has been recently demonstrated that induction of CD40 occurs via the activation of NF-κB, and additionally involves the induction of histone acetylation and phosphorylation at the CD40 promoter [73]. Using gene-expression profiling on $CD3^+$ T cells from Hungarian monozygotic MS twins, an altered transcriptional regulatory network involving NF-κB signalling was identified, which reinforces the notion that aberrant NF-κB signalling is an important contributor to MS pathogenesis [155].

TNF-α activates several key genes through the nuclear transcription factor NF-κB, which plays pivotal roles in the regulation of many responses including inflammation and immune reactivity [156,157]. *In vitro* treatments of cell lines with HDIs induce the expression of various genes including IL-6 and IL-8 [140,150]. Intriguingly, several studies have shown that butyrate can suppress NF-κB activation and its related responses [158–161]. It appears that in normal cells butyrate suppresses the secretion of IL-8, but enhances its expression in cancer cells [162]. HDIs may therefore be able to inhibit NF-κB-mediated inflammatory responses in normal cells, an important implication for neurodegenerative conditions. Additional evidence in support of this notion came from a study in Crohn's disease which demonstrated that butyrate was capable of inhibiting NF-κB-mediated inflammation and reducing pro-inflammatory cytokine mRNA expression [163]. In the EAE mouse model of MS, treatment with a standard MS intervention, IFN-β, resulted in a reduction of active NF-κB subunits in treated animals, indicating that this may be one of the mechanisms by which IFN-β exerts its anti-inflammatory effects in MS [164].

Finally, an important study has demonstrated that inhibition of NF-κB in the central nervous system ameliorates MS symptoms in the EAE mouse model. This seminal study provides strong evidence that NF-κB-dependent gene expression is critical for inflammation and tissue damage in MS [165].

23.11 HATs, HDACs and ER stress

The maintenance of cellular homeostasis requires that a cell be able to sense cellular stress, and react appropriately to alleviate its consequences. There are many ways by which endogenous or exogenous stress can occur in a cell, including pathogenic infection, chemical insult, genetic mutation, nutrient deprivation and even normal differentiation.

Within the cell, the ER is a large organelle consisting of a network of interconnected, closed membrane-bound vesicles. It is the site for the synthesis, folding and modification of secretory and cell-surface proteins and provides many essential cellular functions, including the production of cell membrane components, proteins, lipids and sterols [166]. Only correctly folded proteins are transported out of the ER, and incompletely folded proteins are retained within the ER to either complete the folding process or to be targeted for destruction [167]. Due to its importance, this organelle is vital to cellular homeostasis. However, many conditions can interfere with normal ER function and lead to a situation called ER stress. A recent study has linked the increased expression of ER sress-related proteins in MS lesions, with the greatest expression observed at the edges of chronic active lesions [168].

Thus, ER stress can arise from a disturbance in protein folding which results in an accumulation of unfolded or misfolded proteins within the organelle [169]. If this occurs, the ER has evolved specialized mechanisms that promote proper folding of aberrant protein, thus preventing its aggregation. Simply put, when ER stress occurs, the ER responds by inducing the expression of specific genes in an attempt to restore normal function and to maintain cellular homeostasis [170]. The principal mechanisms of stress response involve (1) protein degradation, (2) endoplasmic overload responses, (3) unfolded protein responses and (4) cellular death. This four-stage model helps to explain the role of ER stress in the onset of clinical manifestations. Two ER stress-induced signal transduction pathways have been described: the unfolded protein responses [171] and the endoplasmic overload responses [172]. The function of these pathways is to attempt to re-establish normal ER function [173]. However, excessive or prolonged ER stress may overwhelm the cell, and subsequently inititate apoptosis [174]. The evidence linking HATs/HDACs to ER stress is not as well established as that for inflammation, with most studies utilizing HDIs. However, in a recent study in hepatocytes on Mallory body (cytokeratin aggresome) formation, decreased HAT and increased HDAC activity was observed [175]. In a similar model of oxidative stress-induced inclusion formation, treatment of cells with 4-phenylbutyrate was found to alleviate formation of these inclusions [176].

Direct physical evidence for the association of HATs and HDACs with critical regulatory elements within the ER stress pathway is emerging. C/EBP homologous protein (CHOP) is an ER-stress-inducible protein which plays a critical role in regulating programmed cell death. CHOP has recently been shown to directly associate with the HAT p300, and inhibition of HDACs prevents its degradation [177]. Chromatin immunoprecipitation strategies have also shown that p300 binds to the promoter of GRP78/BiP, a prosurvival ER chaperone gene induced under conditions of ER stress [178]. In similar studies examining various promoters of ER-stress-responsive genes, histone H4 hyper-acetylation was observed following the induction of ER stress [179].

The B lymphocyte-induced maturation protein-1 (BLIMP-1) is a protein associated with cellular stress. In some cellular models, BLIMP-1 is rapidly up-regulated during the unfolded protein response [180]. This repressor protein has been shown to directly associate with HDACs to repress transcription [104], and this may indicate that BLIMP-1 may utilize HDACs to repress important genes during ER stress.

Data from *in vitro* studies are emerging demonstrating the efficacy of the HDI sodium 4-phenylbutyrate in relieving ER stress in cell-line models of cystic fibrosis [181], and mutant α1-ATZ liver disease [182]. Since these initial observations, several studies have shown that sodium 4-phenylbutyrate may act as a chemical chaperone to relieve ER stress induced in models of ischaemia [183,184]. Similar data have emerged for models of cataract formation [185], Parkinson's disease [186], retinitis pigmentosa [187], glaucoma [188] and cystic fibrosis [189].

In diabetes, sodium 4-phenylbutyrate has also been shown to both relieve ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. This occurs by the restoration of systemic insulin sensitivity, resolution of fatty liver disease and enhancement of insulin action in liver, muscle and adipose tissues [190].

ER stress in liver-induced ischaemia has been shown to be ameliorated by treatments with sodium 4-phenylbutyrate [184]. This compound has also been shown to alleviate oxidative stress-induced ER stress in cultured hepatocytes and hepatoma cells [176], and prevents ER-stress-mediated aggregate formation in a model of hereditary haemochromatosis [191].

Valproic acid, another HDI, has also been shown to have potential in the treatment of ER stress. Initial studies demonstrated that treatment of cells with valproic acid caused the up-regulation of GRP78/BiP, a key ER-mediated chaperone [192]. Subsequent studies confirmed that valproic acid could increase the expression of additional important ER stress proteins, GRP94 and calreticulin [193–195]. Valproic acid has also been shown to protect against oxidative stress-induced protein damage, and had provided neuroprotection in the same model system [196–198].

23.12 Clinical trials and caveats of HDIs

Several HDIs have undergone both phase I/phase II clinical trials as anticancer agents [199], and as previously mentioned SAHA (Vorinostat) has been approved by

the US Food and Drug Administration for the treatment of cutaneous T cell lymphoma. Several of these trials have included, or currently include, patients with neurodegerative diseases, including MS, and as these trials proceed a greater understanding of the potential side effects and dose-limiting toxicities of these drugs will emerge. For the most part, these first-generation inhibitors have shown well-tolerated safety profiles.

Intriguingly, HDIs have been shown to be ineffective in causing apoptosis in cancer cell line models. This was shown to be due to the transcriptional activation of NF-κB through the Akt pathway, with up-regulation of IL-8, Bcl-x_L and MMP-9 transcripts [200]. A follow-up study using SAHA (Vorinostat) has shown that this drug stimulates NF-κB transcription through a signalling cascade involving the activation of Akt and the p300 acetyltransferase [201]. As such these studies provide evidence that HDIs such as SAHA not only inhibit deacetylase activity but also stimulate active NF-κB transcription and cell survival through signalling pathways involving Akt and increased p300 acetyltransferase activity. This may have important implications in the use of HDACs in the treatment of cancer as they may promote cancer cell survival; in contrast, they may have important therapeutic implications in neurodegenerative disease as they may promote the survival or protection of neuronal cells.

Despite the evidence strongly suggesting that HDIs may have an important therapeutic role in the treatment of neurodegenerative disease, some caveats exist which temper this notion. For instance, HDIs have also been shown to up-regulate NF-κB-driven pro-inflammatory cascades [202,203], albeit within a neural setting. Nevertheless, this may also be true for multiple tissue types. In addition, HDIs have also been shown to activate NF-κB, and to sustain the activation of NF-κB by delaying IκBα mRNA resynthesis [204–206]. Again, also within the clinical setting, phenylbutyrate was shown to be ineffective in relieving ER stress in patients with α1-antitrypsin deficiency [207].

An early study utilizing T cell lines found that TSA down-regulated IL-2, suppressed the expression of the T cell receptor ζ chain gene and up-regulated the expression of its homologous gene Fcε receptor I γ chain. These and other data were considered to be reminiscent of the signalling aberrations that have been described in patients with systemic lupus erythematosus [208], and as such HDIs such as TSA may aggravate rather than ameliorate autoimmune conditions. Finally, a phase II clinical trial of phenylbutyrate in patients with spinal muscular atrophy was shown to be ineffective [209], and indicates that much more work is required to realize the potential of HDIs in the neuronal setting.

Other HDIs also continue to show potential in the treatment of neurodegenerative conditions. In a phase I trial of valproic acid on patients with human T-lymphotropic virus type 1 (HTLV-1), which is responsible for HTLV-associated myelopathy/tropical spastic paraparesis, clinical efficacy was observed. Sixteen patients were recruited and valproic acid was administrated orally at a maximal dose of 20 mg/kg per day. There was a significant drop in patient viral load from month 0 to month 3. For the first time,

this trial provides evidence that valproic acid leads to depletion of HTLV-1-infected cells *in vivo* [210].

23.13 Do HDIs target genes or help chaperone activity as their primary response?

Despite these caveats, a plethora of studies have clearly shown that HDIs can reactivate or alter gene expression. However, in addition, several studies now indicate that HDACs and HDIs may play important roles in regulating chaperone expression and function. This has important implications in conditions such as MS where aberrant misfolding of proteins can result in ER stress. This can be highlighted by the fact that HDAC6 is recognized as a leading regulator of cellular efforts to counteract the deleterious effects of misfolded protein accumulation [211–214]. Inhibition or depletion of HDAC6 leads to an induction of heat shock protein 90 (HSP90) acetylation, inhibiting its chaperone activity and eliciting cellular responses [215–220]. The class I HDACs (HDACs 1–3) have been shown to associate with the ATP-dependent chaperone HSP70, and this association enhances deacetylase catalytic activity [221].

Emerging data clearly link the use of HDIs in the relief of ER stress in cell-line models. Many of these studies have utilized sodium 4-phenylbutyrate. In a hepatocyte cell-line model of oxidative stress-induced ER stress, treatment of cells with sodium 4-phenylbutyrate was found to alleviate the ER stress [176]. Other examples of the benefits of sodium 4-phenylbutyrate as a chemical chaperone have been described for relieving apoptosis and/or ER stress in models of eye disease [187,188], rescue of defective trafficking of nephrin in kidney [222], rescue of protein trafficking in the lysosomal storage disorder Fabry disease [223], correction of autodominant hypoparathyroidism-induced apoptosis [224] and ER-stress-mediated programmed cell death in *Arabadopsis* [225].

Other evidence for the potential of HDIs influencing chaperone-like activities to relieve ER stress is emerging. For example, studies on cystic fibrosis have shown that phenylbutyrate can restore cystic fibrosis transmembrane conductance regulator (CFTR) protein trafficking and function [181,226–233] through the induction of HSP90 [233]. Other lung conditions for which beneficial responses have been observed using HDIs as chemical chaperones include respiratory distress syndrome [234] and emphysema [182].

Benefits accruing to the use of HDIs as chemical chaperones in the liver have also been described. In one instance, sodium 4-phenylbutyrate was used to protect liver cells from ER-stress-mediated apoptosis induced by liver ischaemia [184], to enhance the cell-surface expression and transport capacity of mutated bile-salt-export pumps [235], and to reduce ER-stress-induced formation of Mallory bodies in hepatocytes [176].

Increasing evidence is linking the activities of HDIs to ER stress in obesity. For instance, in a cell-line model, phenylbutyrate has been shown to restore functionality to misfolded low-density lipoprotein receptors, and shuttle them to the cell surface. The

authors concluded that their results indicate that phenylbutyrate did not just solely mediate this response by its ability to induce gene expression of proteins involved in intracellular transport, but could also mediate this effect via a direct chemical chaperone activity [236]. Valproic acid was also shown to protect cells from ER stress-induced lipid accumulation and apoptosis via inhibition of glycogen synthase kinase-3 [237]. In a mouse model of type 2 diabetes, phenylbutyrate was found to reduce ER stress and restore glucose homeostasis in the mutant mice [190]. This critical result underlines the potential importance of HDIs as both regulators of gene expression and as chemical chaperones to dampen down inflammation and to relieve ER stress.

Finally, in the neuronal setting, initial studies on mood-stabilizing drugs such as valproic acid demonstrated increased expression of ER stress proteins in cerebral cortex, hippocampus and neuronal and glial cells [192–195,238,239]. Neuroprotective effects of these HDIs have been observed to protect against ischaemia [183,240,241], malonate toxicity [242], the pesticide and insecticide rotenone [243,244] and the tumour promoter thapsigargin [245], and to relieve ER stress induction in a model of autosomal recessive juvenile parkinsonism [186].

All of the above evidence clearly indicates that HDIs may play positive roles in modulating both ER stress and inflammation, and further underlines the potential importance of these inhibitors as an important therapeutic target in MS.

23.14 Future directions

One critical element which needs to be addressed in future studies examining the efficacy of HDIs in combinatorial trials should include studies to address the importance of timing for the scheduling of HDIs in a clinical trial protocol. For instance, in a recent study of non-small-cell lung cancer *in vitro* it was found that the timing of addition of HDIs was a critical determinant that could cause either cell protection or sensitization to camptothecin, an inhibitor of the DNA enzyme topoisomerase I [246]. If timing of administration is critical, this may have important implications for the treatment of MS episodes. These and other questions remain to be addressed, but it is clear that these inhibitors may play important roles in targeting gene transcription and potentially through alleviating ER stress. Further experiments will be required to functionally address these possibilities.

Another very obvious question which needs to be addressed is the role of other histone post-translational modifications in MS pathogenesis. Histone methylation is one emerging modification which warrants serious study. Several inhibitors targeting PRMTs have been developed [247–251], and may be an indirect way of targeting histone arginine methylation prior to conversion to citrulline [247–251]. Alternatively, inhibitors directed against protein arginine deiminases have also been developed [247–251]. These are important clinical avenues of investigation in the pathogenesis of MS, especially in light of recent studies showing that PRMT4 enhances NF-κB-mediated transcription at the promoters of inflammatory genes [252].

References

1. Ragonese, P., Aridon, P., Salemi, G. *et al.* (2008) Mortality in multiple sclerosis: a review. *European Journal of Neurology* **15**, 123–127.
2. Sobocki, P., Pugliatti, M., Lauer, K. *et al.* (2007) Estimation of the cost of MS in Europe: extrapolations from a multinational cost study. *Multiple Sclerosis* **13**, 1054–1064.
3. Smith, L.T., Otterson, G.A. and Plass, C. (2007) Unraveling the epigenetic code of cancer for therapy. *Trends in Genetics* **23**, 449–456.
4. Barlesi, F., Giaccone, G., Gallegos-Ruiz, M.I. *et al.* (2007) Global histone modifications predict prognosis of resected non small-cell lung cancer. *Journal of Clinical Oncology* **25**, 4358–4364.
5. Pal, S. and Sif, S. (2007) Interplay between chromatin remodelers and protein arginine methyltransferases. *Journal of Cellular Physiology* **213**, 306–315.
6. Wang, Y., Wysocka, J., Sayegh, J. *et al.* (2004) Human PAD4 regulates histone arginine methylation levels via demethylimination. *Science* **306**, 279–283.
7. Mastronardi, F.G., Wood, D.D., Mei, J. *et al.* (2006) Increased citrullination of histone H3 in multiple sclerosis brain and animal models of demyelination: a role for tumor necrosis factor-induced peptidylarginine deiminase 4 translocation. *Journal of Neuroscience* **26**, 11387–11396.
8. Wood, D.D., Ackerley, C.A., Brand, B. *et al.* (2008) Myelin localization of peptidylarginine deiminases 2 and 4: comparison of PAD2 and PAD4 activities. *Laboratory Investigation* **88**, 354–364.
9. Mann, B.S., Johnson, J.R., Cohen, M.H. *et al.* (2007) FDA approval summary: vorinostat for treatment of advanced primary cutaneous T-cell lymphoma. *Oncologist* **12**, 1247–1252.
10. Sarkander, H.I., Fleischer-Lambropoulos, H. and Brade, W.P. (1975) A comparative study of histone acetylation in neuronal and glial nuclei enriched rat brain fractions. *FEBS Letters* **52**, 40–43.
11. Liu, H., Hu, Q., Kaufman, A. *et al.* (2008) Developmental expression of histone deacetylase 11 in the murine brain. *Journal of Neuroscience Research* **86**, 537–543.
12. Lakowski, B., Roelens, I. and Jacob, S. (2006) CoREST-like complexes regulate chromatin modification and neuronal gene expression. *Journal of Molecular Neuroscience* **29**, 227–239.
13. Weaver, I.C., Cervoni, N., Champagne, F.A. *et al.* (2004) Epigenetic programming by maternal behavior. *Nature Neuroscience* **7**, 847–854.
14. Renthal, W., Maze, I., Krishnan, V. *et al.* (2007) Histone deacetylase 5 epigenetically controls behavioral adaptations to chronic emotional stimuli. *Neuron* **56**, 517–529.
15. Seth, K.A. and Majzoub, J.A. (2001) Repressor element silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) can act as an enhancer as well as a repressor of corticotropin-releasing hormone gene transcription. *Journal of Biological Chemistry* **276**, 13917–13923.
16. Belyaev, N.D., Wood, I.C., Bruce, A.W. *et al.* (2004) Distinct RE-1 silencing transcription factor-containing complexes interact with different target genes. *Journal of Biological Chemistry* **279**, 556–561.
17. Chen, Z.F., Paquette, A.J. and Anderson, D.J. (1998) NRSF/REST is required in vivo for repression of multiple neuronal target genes during embryogenesis. *Nature Genetics* **20**, 136–142.
18. Paquette, A.J., Perez, S.E. and Anderson, D.J. (2000) Constitutive expression of the neuron-restrictive silencer factor (NRSF)/REST in differentiating neurons disrupts neuronal gene expression and causes axon pathfinding errors in vivo. *Proceedings of the National Academy of Sciences USA* **97**, 12318–12323.
19. Bahn, S., Mimmack, M., Ryan, M. *et al.* (2002) Neuronal target genes of the neuron-restrictive silencer factor in neurospheres derived from fetuses with Down's syndrome: a gene expression study. *Lancet* **359**, 310–315.

20. Thiel, G., Lietz, M. and Cramer, M. (1998) Biological activity and modular structure of RE-1-silencing transcription factor (REST), a repressor of neuronal genes. *Journal of Biological Chemistry* **273**, 26891–26899.
21. Tapia-Ramirez, J., Eggen, B.J., Peral-Rubio, M.J. *et al.* (1997) A single zinc finger motif in the silencing factor REST represses the neural-specific type II sodium channel promoter. *Proceedings of the National Academy of Sciences USA* **94**, 1177–1182.
22. Grimes, J.A., Nielsen, S.J., Battaglioli, E. *et al.* (2000) The co-repressor mSin3A is a functional component of the REST-CoREST repressor complex. *Journal of Biological Chemistry* **275**, 9461–9467.
23. Huang, Y., Myers, S.J. and Dingledine, R. (1999) Transcriptional repression by REST: recruitment of Sin3A and histone deacetylase to neuronal genes. *Nature Neuroscience* **2**, 867–872.
24. Naruse, Y., Aoki, T., Kojima, T. *et al.* (1999) Neural restrictive silencer factor recruits mSin3 and histone deacetylase complex to repress neuron-specific target genes. *Proceedings of the National Academy of Sciences USA* **96**, 13691–13696.
25. Roopra, A., Sharling, L., Wood, I.C. *et al.* (2000) Transcriptional repression by neuron-restrictive silencer factor is mediated via the SIN3-histone deacetylase complex. *Molecular & Cellular Biology* **20**, 2147–2157.
26. Andres, M.E., Burger, C., Peral-Rubio, M.J. *et al.* (1999) CoREST: a functional corepressor required for regulation of neural- specific gene expression. *Proceedings of the National Academy of Sciences USA* **96**, 9873–9878.
27. You, A., Tong, J.K., Grozinger, C.M. *et al.* (2001) CoREST is an integral component of the CoREST-human histone deacetylase complex. *Proceedings of the National Academy of Sciences USA* **98**, 1454–1458.
28. Ballas, N., Grunseich, C., Lu, D.D. *et al.* (2005) REST and its corepressors mediate plasticity of neuronal gene chromatin throughout neurogenesis. *Cell* **121**, 645–657.
29. Battaglioli, E., Andres, M.E., Rose, D.W. *et al.* (2002) REST repression of neuronal genes requires components of the hSWI.SNF complex. *Journal of Biological Chemistry* **277**, 41038–41045.
30. Watanabe, H., Mizutani, T., Haraguchi, T. *et al.* (2006) SWI/SNF complex is essential for NRSF-mediated suppression of neuronal genes in human nonsmall cell lung carcinoma cell lines. *Oncogene* **25**, 470–479.
31. Wang, J., Scully, K., Zhu, X. *et al.* (2007) Opposing LSD1 complexes function in developmental gene activation and repression programmes. *Nature* **446**, 882–887.
32. Horio, Y., Hisahara, S. and Sakamoto, J. (2003) Functional analysis of SIR2. *Nippon Yakurigaku Zasshi* **122** (suppl.), 30P–32P.
33. Kyrylenko, S., Kyrylenko, O., Suuronen, T. *et al.* (2003) Differential regulation of the Sir2 histone deacetylase gene family by inhibitors of class I and II histone deacetylases. *Cellular & Molecular Life Sciences* **60**, 1990–1997.
34. Ajamian, F., Suuronen, T., Salminen, A. *et al.* (2003) Upregulation of class II histone deacetylases mRNA during neural differentiation of cultured rat hippocampal progenitor cells. *Neuroscience Letters* **346**, 57–60.
35. Hoshino, M., Tagawa, K., Okuda, T. *et al.* (2003) Histone deacetylase activity is retained in primary neurons expressing mutant huntingtin protein. *Journal of Neurochemistry* **87**, 257–267.
36. Brunet, A., Sweeney, L.B., Sturgill, J.F. *et al.* (2004) Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* **303**, 2011–2015.
37. Linseman, D.A., Bartley, C.M., Le, S.S. *et al.* (2003) Inactivation of the myocyte enhancer factor-2 repressor histone deacetylase-5 by endogenous Ca(2+)//calmodulin-dependent kinase II promotes depolarization-mediated cerebellar granule neuron survival. *Journal of Biological Chemistry* **278**, 41472–41481.

38. Sharma, K., Sheng, H.Z., Lettieri, K. *et al.* (1998) LIM homeodomain factors Lhx3 and Lhx4 assign subtype identities for motor neurons. *Cell* **95**, 817–828.
39. Bach, I., Rodriguez-Esteban, C., Carriere, C. *et al.* (1999) RLIM inhibits functional activity of LIM homeodomain transcription factors via recruitment of the histone deacetylase complex. *Nature Genetics* **22**, 394–399.
40. Boutillier, A.L., Trinh, E. and Loeffler, J.P. (2002) Constitutive repression of E2F1 transcriptional activity through HDAC proteins is essential for neuronal survival. *Annals of the New York Academy of Science* **973**, 438–442.
41. Konishi, Y. and Bonni, A. (2003) The E2F-Cdc2 cell-cycle pathway specifically mediates activity deprivation-induced apoptosis of postmitotic neurons. *Journal of Neuroscience* **23**, 1649–1658.
42. Hou, S.T., Callaghan, D., Fournier, M.C. *et al.* (2000) The transcription factor E2F1 modulates apoptosis of neurons. *Journal of Neurochemistry* **75**, 91–100.
43. Wu, L., Timmers, C., Maiti, B. *et al.* (2001) The E2F1-3 transcription factors are essential for cellular proliferation. *Nature* **414**, 457–462.
44. Iglesias, A.H., Camelo, S., Hwang, D. *et al.* (2004) Microarray detection of E2F pathway activation and other targets in multiple sclerosis peripheral blood mononuclear cells. *Journal of Neuroimmunology* **150**, 163–177.
45. Camelo, S., Iglesias, A.H., Hwang, D. *et al.* (2005) Transcriptional therapy with the histone deacetylase inhibitor trichostatin A ameliorates experimental autoimmune encephalomyelitis. *Journal of Neuroimmunology* **164**, 10–21.
46. Suzuki, T., Yokozaki, H., Kuniyasu, H. *et al.* (2000) Effect of trichostatin A on cell growth and expression of cell cycle- and apoptosis-related molecules in human gastric and oral carcinoma cell lines. *International Journal of Cancer* **88**, 992–997.
47. Said, T.K., Moraes, R.C., Sinha, R. *et al.* (2001) Mechanisms of suberoylanilide hydroxamic acid inhibition of mammary cell growth. *Breast Cancer Research* **3**, 122–133.
48. Dangond, F., Hafler, D.A., Tong, J.K. *et al.* (1998) Differential display cloning of a novel human histone deacetylase (HDAC3) cDNA from PHA-activated immune cells. *Biochemical & Biophysical Research Communications* **242**, 648–652.
49. Ryu, H., Lee, J., Olofsson, B.A. *et al.* (2003) Histone deacetylase inhibitors prevent oxidative neuronal death independent of expanded polyglutamine repeats via an Sp1-dependent pathway. *Proceedings of the National Academy of Sciences USA* **100**, 4281–4286.
50. Phiel, C.J., Zhang, F., Huang, E.Y. *et al.* (2001) Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *Journal of Biological Chemistry* **276**, 36734–36741.
51. Gottlicher, M., Minucci, S., Zhu, P. *et al.* (2001) Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO Journal* **20**, 6969–6978.
52. Jeong, M.R., Hashimoto, R., Senatorov, V.V. *et al.* (2003) Valproic acid, a mood stabilizer and anticonvulsant, protects rat cerebral cortical neurons from spontaneous cell death: a role of histone deacetylase inhibition. *FEBS Letters* **542**, 74–78.
53. Humphrey, G.W., Wang, Y.H., Hirai, T. *et al.* (2007) Complementary roles for histone deacetylases 1, 2, and 3 in differentiation of pluripotent stem cells. *Differentiation* **76**, 348–356.
54. Balasubramaniyan, V., Boddeke, E., Bakels, R. *et al.* (2006) Effects of histone deacetylation inhibition on neuronal differentiation of embryonic mouse neural stem cells. *Neuroscience* **143**, 939–951.
55. Lyssiotis, C.A., Walker, J., Wu, C. *et al.* (2007) Inhibition of histone deacetylase activity induces developmental plasticity in oligodendrocyte precursor cells. *Proceedings of the National Academy of Sciences USA* **104**, 14982–14987.
56. Liu, A., Han, Y.R., Li, J. *et al.* (2007) The glial or neuronal fate choice of oligodendrocyte progenitors is modulated by their ability to acquire an epigenetic memory. *Journal of Neuroscience* **27**, 7339–7343.

57. He, Y., Dupree, J., Wang, J. *et al.* (2007) The transcription factor Yin Yang 1 is essential for oligodendrocyte progenitor differentiation. *Neuron* **55**, 217–230.
58. Sun, G., Yu, R.T., Evans, R.M. *et al.* (2007) Orphan nuclear eceptor TLX recruits histone deacetylases to repress transcription and regulate neural stem cell proliferation. *Proceedings of the National Academy of Sciences USA* **104**, 15282–15287.
59. Su, X., Kameoka, S., Lentz, S. *et al.* (2004) Activation of REST/NRSF target genes in neural stem cells is sufficient to cause neuronal differentiation. *Molecular & Cellular Biology* **24**, 8018–8025.
60. Greenway, D.J., Street, M., Jeffries, A. *et al.* (2007) RE1 silencing transcription factor maintains a repressive chromatin environment in embryonic hippocampal neural stem cells. *Stem Cells* **25**, 354–363.
61. Sun, Y.M., Greenway, D.J., Johnson, R. *et al.* (2005) Distinct profiles of REST interactions with its target genes at different stages of neuronal development. *Molecular Biology of the Cell* **16**, 5630–5638.
62. Gray, S.G. and Teh, B.T. (2001) Histone acetylation/deacetylation and cancer: an ''open'' and ''shut'' case? *Current Molecular Medicine* **1**, 401–429.
63. Abbas, A.K., Murphy, K.M. and Sher, A. (1996) Functional diversity of helper T lymphocytes. *Nature* **383**, 787–793.
64. O'garra, A. (1998) Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* **8**, 275–283.
65. Chitnis, T. (2007) The role of CD4 T cells in the pathogenesis of multiple sclerosis. *International Review of Neurobiology* **79**, 43–72.
66. Morinobu, A., Kanno, Y. and O'shea, J.J. (2004) Discrete roles for histone acetylation in human T helper 1 cell-specific gene expression. *Journal of Biological Chemistry* **279**, 40640–40646.
67. Brogdon, J.L., Xu, Y., Szabo, S.J. *et al.* (2007) Histone deacetylase activities are required for innate immune cell control of Th1 but not Th2 effector cell function. *Blood* **109**, 1123–1130.
68. Shi, Y., Feng, Y., Kang, J. *et al.* (2007) Critical regulation of CD4+ T cell survival and autoimmunity by beta-arrestin 1. *Nature Immunology* **8**, 817–824.
69. Yamashita, M., Kuwahara, M., Suzuki, A. *et al.* (2008) Bmi1 regulates memory CD4 T cell survival via repression of the Noxa gene. *Journal of Experimental Medicine* **205**, 1109–1120.
70. Rott, O., Fleischer, B. and Cash, E. (1994) Interleukin-10 prevents experimental allergic encephalomyelitis in rats. *European Journal of Immunology* **24**, 1434–1440.
71. Cua, D.J., Hutchins, B., Laface, D.M. *et al.* (2001) Central nervous system expression of IL-10 inhibits autoimmune encephalomyelitis. *Journal of Immunology* **166**, 602–608.
72. Laman, J.D., De Boer, M. and Hart, B.A. (1998) CD40 in clinical inflammation: from multiple sclerosis to atherosclerosis. *Developmental Immunology* **6**, 215–222.
73. Qin, H., Wilson, C.A., Lee, S.J. *et al.* (2005) LPS induces CD40 gene expression through the activation of NF-kappaB and STAT-1alpha in macrophages and microglia. *Blood* **106**, 3114–3122.
74. Qin, H., Wilson, C.A., Roberts, K.L. *et al.* (2006) IL-10 inhibits lipopolysaccharide-induced CD40 gene expression through induction of suppressor of cytokine signaling-3. *Journal of Immunology* **177**, 7761–7771.
75. Kuchroo, V.K., Das, M.P., Brown, J.A. *et al.* (1995) B7–1 and B7–2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell* **80**, 707–718.
76. Leonard, J.P., Waldburger, K.E. and Goldman, S.J. (1995) Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. *Journal of Experimental Medicine* **181**, 381–386.
77. Bright, J.J., Musuro, B.F., Du, C. *et al.* (1998) Expression of IL-12 in CNS and lymphoid organs of mice with experimental allergic encephalitis. *Journal of Neuroimmunology* **82**, 22–30.

78. Lu, J., Sun, H., Wang, X. *et al.* (2005) Interleukin-12 p40 promoter activity is regulated by the reversible acetylation mediated by HDAC1 and p300. *Cytokine* **31**, 46–51.
79. Dangond, F. and Gullans, S.R. (1998) Differential expression of human histone deacetylase mRNAs in response to immune cell apoptosis induction by trichostatin A and butyrate. *Biochemical & Biophysical Research Communications* **247**, 833–837.
80. Saemann, M.D., Bohmig, G.A., Osterreicher, C.H. *et al.* (2000) Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production. *FASEB Journal* **14**, 2380–2382.
81. O'connor, K.C., Bar-or, A. and Hafler, D.A. (2001) The neuroimmunology of multiple sclerosis: possible roles of T and B lymphocytes in immunopathogenesis. *Journal of Clinical Immunology* **21**, 81–92.
82. Takahashi, I., Miyaji, H., Yoshida, T. *et al.* (1996) Selective inhibition of IL-2 gene expression by trichostatin A, a potent inhibitor of mammalian histone deacetylase. *Journal of Antibiotics* **49**, 453–457.
83. Matsuoka, H., Fujimura, T., Mori, H. *et al.* (2007) Mechanism of HDAC inhibitor FR235222-mediated IL-2 transcriptional repression in Jurkat cells. *International Immunopharmacology* **7**, 1422–1432.
84. Koyama, Y., Adachi, M., Sekiya, M. *et al.* (2000) Histone deacetylase inhibitors suppress IL-2-mediated gene expression prior to induction of apoptosis. *Blood* **96**, 1490–1495.
85. Matsuoka, H., Fujimura, T., Hayashi, M. *et al.* (2007) Disruption of HDAC4/N-CoR complex by histone deacetylase inhibitors leads to inhibition of IL-2 gene expression. *Biochemical Pharmacology* **74**, 465–476.
86. Tao, R., De Zoeten, E.F., Ozkaynak, E. *et al.* (2007) Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nature Medicine* **13**, 1299–1307.
87. Li, B., Samanta, A., Song, X. *et al.* (2007) FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. *Proceedings of the National Academy of Sciences USA* **104**, 4571–4576.
88. Gilbert, K.M. and Weigle, W.O. (1993) Th1 cell anergy and blockade in G1a phase of the cell cycle. *Journal of Immunology* **151**, 1245–1254.
89. Gilbert, K.M., Wahid, R., Fecher, N.P. *et al.* (2000) Potential clinical use of butyric acid derivatives to induce antigen- specific T cell inactivation. *Journal of Pharmacology and Experimental Therapeutics* **294**, 1146–1153.
90. Edens, R.E., Dagtas, S. and Gilbert, K.M. (2006) Histone deacetylase inhibitors induce antigen specific anergy in lymphocytes: a comparative study. *International Immunopharmacology* **6**, 1673–1681.
91. Kaneko, T., Hosokawa, H., Yamashita, M. *et al.* (2007) Chromatin remodeling at the Th2 cytokine gene loci in human type 2 helper T cells. *Molecular Immunology* **44**, 2249–2256.
92. Kanazawa, S. and Peterlin, B.M. (2001) Combinations of dominant-negative class II transactivator, p300 or CDK9 proteins block the expression of MHC II genes. *International Immunology* **13**, 951–958.
93. Fontes, J.D., Kanazawa, S., Jean, D. *et al.* (1999) Interactions between the class II transactivator and CREB binding protein increase transcription of major histocompatibility complex class II genes. *Molecular & Cellular Biology* **19**, 941–947.
94. Masternak, K., Muhlethaler-Mottet, A., Villard, J. *et al.* (2000) CIITA is a transcriptional coactivator that is recruited to MHC class II promoters by multiple synergistic interactions with an enhanceosome complex. *Genes & Development* **14**, 1156–1166.
95. Raval, A., Howcroft, T.K., Weissman, J.D. *et al.* (2001) Transcriptional coactivator, CIITA, is an acetyltransferase that bypasses a promoter requirement for TAF(II)250. *Molecular Cell* **7**, 105–115.

96. Masternak, K. and Reith, W. (2002) Promoter-specific functions of CIITA and the MHC class II enhanceosome in transcriptional activation. *EMBO Journal* **21**, 1379–1388.
97. Zika, E., Greer, S.F., Zhu, X.S. *et al.* (2003) Histone deacetylase 1/mSin3A disrupts gamma interferon-induced CIITA function and major histocompatibility complex class II enhanceosome formation. *Molecular & Cellular Biology* **23**, 3091–3102.
98. Mckinsey, T.A., Kuwahara, K., Bezprozvannaya, S. *et al.* (2006) Class II histone deacetylases confer signal responsiveness to the ankyrin-repeat proteins ANKRA2 and RFXANK. *Molecular Biology of the Cell* **17**, 438–447.
99. Wang, A.H., Gregoire, S., Zika, E. *et al.* (2005) Identification of the ankyrin repeat proteins ANKRA and RFXANK as novel partners of class IIa histone deacetylases. *Journal of Biological Chemistry* **280**, 29117–29127.
100. Piskurich, J.F., Lin, K.I., Lin, Y. *et al.* (2000) BLIMP-I mediates extinction of major histocompatibility class II transactivator expression in plasma cells. *Nature Immunology* **1**, 526–532.
101. Ghosh, N., Gyory, I., Wright, G. *et al.* (2001) Positive regulatory domain I binding factor 1 silences class II transactivator expression in multiple myeloma cells. *Journal of Biological Chemistry* **276**, 15264–15268.
102. Chen, G., Fernandez, J., Mische, S. *et al.* (1999) A functional interaction between the histone deacetylase Rpd3 and the corepressor groucho in *Drosophila* development. *Genes & Development* **13**, 2218–2230.
103. Ren, B., Chee, K.J., Kim, T.H. *et al.* (1999) PRDI-BF1/Blimp-1 repression is mediated by corepressors of the Groucho family of proteins. *Genes & Development* **13**, 125–137.
104. Yu, J., Angelin-Duclos, C., Greenwood, J. *et al.* (2000) Transcriptional repression by blimp-1 (PRDI-BF1) involves recruitment of histone deacetylase. *Molecular & Cellular Biology* **20**, 2592–2603.
105. Osborne, A., Zhang, H., Yang, W.M. *et al.* (2001) Histone deacetylase activity represses gamma interferon-inducible HLA-DR gene expression following the establishment of a DNase I-hypersensitive chromatin conformation. *Molecular & Cellular Biology* **21**, 6495–6506.
106. Magner, W.J., Kazim, A.L., Stewart, C. *et al.* (2000) Activation of MHC class I, II, and CD40 gene expression by histone deacetylase inhibitors. *Journal of Immunology* **165**, 7017–7024.
107. Brockmann, D., Lehmkuhler, O., Schmucker, U. *et al.* (2001) The histone acetyltransferase activity of PCAF cooperates with the brahma/SWI2-related protein BRG-1 in the activation of the enhancer A of the MHC class I promoter. *Gene* **277**, 111–120.
108. Williams, K., Ulvestad, E. and Antel, J.P. (1994) B7/BB-1 antigen expression on adult human microglia studied in vitro and in situ. *European Journal of Immunology* **24**, 3031–3037.
109. Windhagen, A., Newcombe, J., Dangond, F. *et al.* (1995) Expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. *Journal of Experimental Medicine* **182**, 1985–1996.
110. Genc, K., Dona, D.L. and Reder, A.T. (1997) Increased CD80 (+) B cells in active multiple sclerosis and reversal by interferon beta-1b therapy. *Journal of Clinical Investigation* **99**, 2664–2671.
111. Bohmig, G.A., Krieger, P.M., Saemann, M.D. *et al.* (1997) n-butyrate downregulates the stimulatory function of peripheral blood- derived antigen-presenting cells: a potential mechanism for modulating T-cell responses by short-chain fatty acids. *Immunology* **92**, 234–243.
112. Zlokovic, B.V. (2008) The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron* **57**, 178–201.
113. Yong, V.W., Zabad, R.K., Agrawal, S. *et al.* (2007) Elevation of matrix metalloproteinases (MMPs) in multiple sclerosis and impact of immunomodulators. *Journal of the Neurological Sciences* **259**, 79–84.

114. Maeda, A. and Sobel, R.A. (1996) Matrix metalloproteinases in the normal human central nervous system, microglial nodules, and multiple sclerosis lesions. *Journal of Neuropathology and Experimental Neurology* **55**, 300–309.
115. Fiorino, A.S. and Zvibel, I. (1996) Disruption of cell-cell adhesion in the presence of sodium butyrate activates expression of the 92 kDa type IV collagenase in MDCK cells. *Cell Biology International* **20**, 489–499.
116. Kim, M.S., Son, M.W., Kim, W.B. *et al.* (2000) Apicidin, an inhibitor of histone deacetylase, prevents H-ras-induced invasive phenotype. *Cancer Letters* **157**, 23–30.
117. Vinodhkumar, R., Song, Y.S., Ravikumar, V. *et al.* (2007) Depsipeptide a histone deacetlyase inhibitor down regulates levels of matrix metalloproteinases 2 and 9 mRNA and protein expressions in lung cancer cells (A549). *Chemico-biological Interactions* **165**, 220–229.
118. Aparicio, T., Kermorgant, S., Lewin, M.J. *et al.* (1998) Effects of HGF on the production of matrix metalloproteinases by colonic cancer cells DHD/K12. *Comptes Rendus des Séances de la Société de Biologie et de ses Filiales* **192**, 311–315.
119. Liu, L.-T., Chang, H.-C., Chiang, L.-C. *et al.* (2003) Histone deacetylase inhibitor up-regulates RECK to inhibit MMP-2 activation and cancer cell invasion. *Cancer Research* **63**, 3069–3072.
120. Pender, S.L., Quinn, J.J., Sanderson, I.R. *et al.* (2000) Butyrate upregulates stromelysin-1 production by intestinal mesenchymal cells. *American Journal of Physiology Gastrointestinal and Liver Physiology* **279**, G918–G924.
121. Nasu, Y., Nishida, K., Miyazawa, S. *et al.* (2008) Trichostatin A, a histone deacetylase inhibitor, suppresses synovial inflammation and subsequent cartilage destruction in a collagen antibody-induced arthritis mouse model. *Osteoarthritis Cartilage* **16** (6), 723–732.
122. Emenaker, N.J. and Basson, M.D. (1998) Short chain fatty acids inhibit human (SW1116) colon cancer cell invasion by reducing urokinase plasminogen activator activity and stimulating TIMP-1 and TIMP-2 activities, rather than via MMP modulation. *Journal of Surgical Research* **76**, 41–46.
123. Emenaker, N.J., Calaf, G.M., Cox, D. *et al.* (2001) Short-chain fatty acids inhibit invasive human colon cancer by modulating uPA, TIMP-1, TIMP-2, mutant p53, Bcl-2, Bax, p21 and PCNA protein expression in an in vitro cell culture model. *Journal of Nutrition* **131** 3041S–3046s.
124. Thelen, P., Schweyer, S., Hemmerlein, B. *et al.* (2004) Expressional changes after histone deacetylase inhibition by valproic acid in LNCaP human prostate cancer cells. *International Journal of Oncology* **24**, 25–31.
125. Gagnon, J., Shaker, S., Primeau, M. *et al.* (2003) Interaction of 5-aza-2′-deoxycytidine and depsipeptide on antineoplastic activity and activation of 14–3-3sigma, E-cadherin and tissue inhibitor of metalloproteinase 3 expression in human breast carcinoma cells. *Anticancer Drugs* **14**, 193–202.
126. Qian, X., Ara, G., Mills, E. *et al.* (2008) Activity of the histone deacetylase inhibitor belinostat (PXD101) in preclinical models of prostate cancer. *International Journal of Cancer* **122**, 1400–1410.
127. Young, D.A., Billingham, O., Sampieri, C.L. *et al.* (2005) Differential effects of histone deacetylase inhibitors on phorbol ester- and TGF-beta1 induced murine tissue inhibitor of metalloproteinases-1 gene expression. *FEBS Journal* **272**, 1912–1926.
128. Perwez Hussain, S. and Harris, C.C. (2007) Inflammation and cancer: an ancient link with novel potentials. *International Journal of Cancer* **121**, 2373–2380.
129. Karin, M. (2006) Nuclear factor-kappaB in cancer development and progression. *Nature* **441**, 431–436.
130. Qatanani, M. and Lazar, M.A. (2007) Mechanisms of obesity-associated insulin resistance: many choices on the menu. *Genes & Development* **21**, 1443–1455.

131. Esiri, M.M. (2007) The interplay between inflammation and neurodegeneration in CNS disease. *Journal of Neuroimmunology* **184**, 4–16.
132. Moenner, M., Pluquet, O., Bouchecareilh, M. *et al.* (2007) Integrated endoplasmic reticulum stress responses in cancer. *Cancer Research* **67**, 10631–10634.
133. Eizirik, D.L., Cardozo, A.K. and Cnop, M. (2007) The role for endoplasmic reticulum stress in diabetes mellitus. *Endocrine Reviews* **29**, 42–61.
134. Uehara, T. (2007) Accumulation of misfolded protein through nitrosative stress linked to neurodegenerative disorders. *Antioxidants & Redox Signaling* **9**, 597–601.
135. Wei, H., Kim, S.J., Zhang, Z. *et al.* (2007) ER and oxidative stresses are common mediators of apoptosis in both neurodegenerative and non-neurodegenerative lysosomal storage disorders and are alleviated by chemical chaperones. *Human Molecular Genetics* **17**, 469–477.
136. Gerritsen, M.E., Williams, A.J., Neish, A.S. *et al.* (1997) CREB-binding protein/p300 are transcriptional coactivators of p65. *Proceedings of the National Academy of Sciences USA* **94**, 2927–2932.
137. Perkins, N.D., Felzien, L.K., Betts, J.C. *et al.* (1997) Regulation of NF-kappaB by cyclin-dependent kinases associated with the p300 coactivator. *Science* **275**, 523–527.
138. Wadgaonkar, R., Phelps, K.M., Haque, Z. *et al.* (1999) CREB-binding protein is a nuclear integrator of nuclear factor-kappaB and p53 signaling. *Journal of Biological Chemistry* **274**, 1879–1882.
139. Sirianni, N., Naidu, S., Pereira, J. *et al.* (1998) Rett syndrome: confirmation of X-linked dominant inheritance, and localization of the gene to Xq28. *American Journal of Human Genetics* **63**, 1552–1558.
140. Ashburner, B.P., Westerheide, S.D. and Baldwin, A.S. Jr. (2001) The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. *Molecular & Cellular Biology* **21**, 7065–7077.
141. Yeung, F., Hoberg, J.E., Ramsey, C.S. *et al.* (2004) Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. *EMBO Journal* **23**, 2369–2380.
142. Hoberg, J.E., Popko, A.E., Ramsey, C.S. *et al.* (2006) IkappaB kinase alpha-mediated derepression of SMRT potentiates acetylation of RelA/p65 by p300. *Molecular & Cellular Biology* **26**, 457–471.
143. Hoberg, J.E., Yeung, F. and Mayo, M.W. (2004) SMRT derepression by the IkappaB kinase alpha: a prerequisite to NF-kappaB transcription and survival. *Molecular Cell* **16**, 245–255.
144. Park, J., Lee, J.H., La, M. *et al.* (2007) Inhibition of NF-kappaB acetylation and its transcriptional activity by Daxx. *Journal of Molecular Biology* **368**, 388–397.
145. Hollenbach, A.D., Mcpherson, C.J. Mientjes, E.J. *et al.* (2002) Daxx and histone deacetylase II associate with chromatin through an interaction with core histones and the chromatin-associated protein Dek. *Journal of Cell Science* **115**, 3319–3330.
146. Kuo, H.Y., Chang, C.C. Jeng, J.C. *et al.* (2005) SUMO modification negatively modulates the transcriptional activity of CREB-binding protein via the recruitment of Daxx. *Proceedings of the National Academy of Sciences USA* **102**, 16973–16978.
147. Esposito, K., Nappo, F. Giugliano, F. *et al.* (2003) Cytokine milieu tends toward inflammation in type 2 diabetes. *Diabetes Care* **26**, 1647.
148. Dandona, P., Aljada, A., Chaudhuri, A. *et al.* (2004) Endothelial dysfunction, inflammation and diabetes. *Reviews in Endocrine & Metabolic Disorders* **5**, 189–197.
149. Dandona, P., Aljada, A. and Bandyopadhyay, A. (2004) Inflammation: the link between insulin resistance, obesity and diabetes. *Trends in Immunology* **25**, 4–7.
150. Vanden Berghe, W., De Bosscher, K., Boone, E. *et al.* (1999) The nuclear factor-kappa B engages CBP/p300 and histone acetyltransferase activity for transcriptional activation of the interleukin-6 gene promoter. *Journal of Biological Chemistry* **274**, 32091–32098.

151. Bettelli, E., Dastrange, M. and Oukka, M. (2005) Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells. *Proceedings of the National Academy of Sciences USA* **102**, 5138–5143.
152. Kwon, H.K., So, J.S., Lee, C.G. *et al.* (2008) Foxp3 induces IL-4 gene silencing by affecting nuclear translocation of NFkappaB and chromatin structure. *Molecular Immunology*. **45**, 3205–3212.
153. Miterski, B., Bohringer, S., Klein, W. *et al.* (2002) Inhibitors in the NFkappaB cascade comprise prime candidate genes predisposing to multiple sclerosis, especially in selected combinations. *Genes and Immunity* **3**, 211–219.
154. Flores, N., Duran, C., Blasco, M.R. *et al.* (2003) NFkappaB and AP-1 DNA binding activity in patients with multiple sclerosis. *Journal of Neuroimmunology* **135**, 141–147.
155. Satoh, J., Illes, Z., Peterfalvi, A. *et al.* (2007) Aberrant transcriptional regulatory network in T cells of multiple sclerosis. *Neuroscience Letters* **422**, 30–33.
156. Ghosh, S., May, M.J. and Kopp, E.B. (1998) NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annual Review of Immunology* **16**, 225–260.
157. Baldwin, Jr, A.S. (1996) The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annual Review of Immunology* **14**, 649–683.
158. Luhrs, H., Gerke, T., Boxberger, F. *et al.* (2001) Butyrate inhibits interleukin-1-mediated nuclear factor-kappa B activation in human epithelial cells. *Digestive Diseases and Sciences* **46**, 1968–1973.
159. Luhrs, H., Gerke, T., Schauber, J. *et al.* (2001) Cytokine-activated degradation of inhibitory kappaB protein alpha is inhibited by the short-chain fatty acid butyrate. *International Journal of Colorectal Disease* **16**, 195–201.
160. Andoh, A., Fujiyama, Y., Shimada, M. *et al.* (2000) Modulation of complement component (C3 and factor B) biosynthesis by a histone deacetylase inhibitor in human intestinal epithelial cells. *International Journal of Molecular Medicine* **6**, 51–54.
161. Andoh, A., Fujiyama, Y., Hata, K. *et al.* (1999) Counter-regulatory effect of sodium butyrate on tumour necrosis factor- alpha (TNF-alpha)-induced complement C3 and factor B biosynthesis in human intestinal epithelial cells. *Clinical and Experimental Immunology* **118**, 23–29.
162. Gibson, P.R., Rosella, O., Wilson, A.J. *et al.* (1999) Colonic epithelial cell activation and the paradoxical effects of butyrate. *Carcinogenesis* **20**, 539–544.
163. Segain, J.P., Raingeard De La Bletiere, D., Bourreille, A. *et al.* (2000) Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. *Gut* **47**, 397–403.
164. Martin-Saavedra, F.M., Flores, N., Dorado, B. *et al.* (2007) Beta-interferon unbalances the peripheral T cell proinflammatory response in experimental autoimmune encephalomyelitis. *Molecular Immunology* **44**, 3597–3607.
165. Van Loo, G., De Lorenzi, R., Schmidt, H. *et al.* (2006) Inhibition of transcription factor NF-kappaB in the central nervous system ameliorates autoimmune encephalomyelitis in mice. *Nature Immunology* **7**, 954–961.
166. Hebert, D.N. and Molinari, M. (2007) In and out of the ER: protein folding, quality control, degradation, and related human diseases. *Physiological Reviews* **87**, 1377–1408.
167. Rapoport, T.A. (2007) Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes. *Nature* **450**, 663–669.
168. Mháille, A.N., Mcquaid, S., Windebank, A. *et al.* (2008) Increased expression of endoplasmic reticulum stress-related signaling pathway molecules in multiple sclerosis lesions. *Journal of Neuropathology and Experimental Neurology* **67**, 200–211.
169. Lai, E., Teodoro, T. and Volchuk, A. (2007) Endoplasmic reticulum stress: signaling the unfolded protein response. *Physiology (Bethesda)* **22**, 193–201.

170. Caruso, M.E. and Chevet, E. (2007) Systems biology of the endoplasmic reticulum stress response. *Subcellular Biochemistry* **43**, 277–298.
171. Ron, D. and Walter, P. (2007) Signal integration in the endoplasmic reticulum unfolded protein response. *Nature Reviews Molecular & Cellular Biology* **8**, 519–529.
172. Pahl, H.L. and Baeuerle, P.A. (1997) The ER-overload response: activation of NF-kappa B. *Trends in Biochemical Sciences* **22**, 63–67.
173. Xu, C., Bailly-Maitre, B. and Reed, J.C. (2005) Endoplasmic reticulum stress: cell life and death decisions. *Journal of Clinical Investigation* **115**, 2656–2664.
174. Rao, R.V., Ellerby, H.M. and Bredesen, D.E. (2004) Coupling endoplasmic reticulum stress to the cell death program. *Cell Death & Differentiation* **11**, 372–380.
175. Bardag-Gorce, F., Dedes, J., French, B.A. *et al.* (2007) Mallory body formation is associated with epigenetic phenotypic change in hepatocytes in vivo. *Experimental and Molecular Pathology* **83**, 160–168.
176. Hanada, S., Harada, M., Kumemura, H. *et al.* (2007) Oxidative stress induces the endoplasmic reticulum stress and facilitates inclusion formation in cultured cells. *Journal of Hepatology* **47**, 93–102.
177. Ohoka, N., Hattori, T., Kitagawa, M. *et al.* (2007) Critical and functional regulation of CHOP (C/EBP homologous protein) through the N-terminal portion. *Journal of Biological Chemistry* **282**, 35687–35694.
178. Baumeister, P., Luo, S., Skarnes, W.C. *et al.* (2005) Endoplasmic reticulum stress induction of the Grp78/BiP promoter: activating mechanisms mediated by YY1 and its interactive chromatin modifiers. *Molecular & Cellular Biology* **25**, 4529–4540.
179. Donati, G., Imbriano, C. and Mantovani, R. (2006) Dynamic recruitment of transcription factors and epigenetic changes on the ER stress response gene promoters. *Nucleic Acids Research* **34**, 3116–3127.
180. Doody, G.M., Stephenson, S. and Tooze, R.M. (2006) BLIMP-1 is a target of cellular stress and downstream of the unfolded protein response. *European Journal of Immunology* **36**, 1572–1582.
181. Rubenstein, R.C. and Zeitlin, P.L. (2000) Sodium 4-phenylbutyrate downregulates Hsc70: implications for intracellular trafficking of DeltaF508-CFTR. *American Journal of Physiology Cellular Physiology* **278**, C259–C267.
182. Burrows, J.A., Willis, L.K. and Perlmutter, D.H. (2000) Chemical chaperones mediate increased secretion of mutant alpha 1-antitrypsin (alpha 1-AT) Z: A potential pharmacological strategy for prevention of liver injury and emphysema in alpha 1-AT deficiency. *Proceedings of the National Academy of Sciences USA* **97**, 1796–1801.
183. Qi, X., Hosoi, T., Okuma, Y. *et al.* (2004) Sodium 4-phenylbutyrate protects against cerebral ischemic injury. *Molecular Pharmacology* **66**, 899–908.
184. Vilatoba, M., Eckstein, C., Bilbao, G. *et al.* (2005) Sodium 4-phenylbutyrate protects against liver ischemia reperfusion injury by inhibition of endoplasmic reticulum-stress mediated apoptosis. *Surgery* **138**, 342–351.
185. Mulhern, M.L., Madson, C.J., Kador, P.F. *et al.* (2007) Cellular osmolytes reduce lens epithelial cell death and alleviate cataract formation in galactosemic rats. *Molecular Vision* **13**, 1397–1405.
186. Kubota, K., Niinuma, Y., Kaneko, M. *et al.* (2006) Suppressive effects of 4-phenylbutyrate on the aggregation of Pael receptors and endoplasmic reticulum stress. *Journal of Neurochemistry* **97**, 1259–1268.
187. Bonapace, G., Waheed, A., Shah, G.N. *et al.* (2004) Chemical chaperones protect from effects of apoptosis-inducing mutation in carbonic anhydrase IV identified in retinitis pigmentosa 17. *Proceedings of the National Academy of Sciences USA* **101**, 12300–12305.

188. Yam, G.H., Gaplovska-Kysela, K., Zuber, C. *et al.* (2007) Sodium 4-phenylbutyrate acts as a chemical chaperone on misfolded myocilin to rescue cells from endoplasmic reticulum stress and apoptosis. *Investigative Ophthalmology & Visual Science* **48**, 1683–1690.
189. Vij, N., Fang, S. and Zeitlin, P.L. (2006) Selective inhibition of endoplasmic reticulum-associated degradation rescues DeltaF508-cystic fibrosis transmembrane regulator and suppresses interleukin-8 levels: therapeutic implications. *Journal of Biological Chemistry* **281**, 17369–17378.
190. Ozcan, U., Yilmaz, E., Ozcan, L. *et al.* (2006) Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* **313**, 1137–1140.
191. De Almeida, S.F., Picarote, G., Fleming, J.V. *et al.* (2007) Chemical chaperones reduce endoplasmic reticulum stress and prevent mutant HFE aggregate formation. *Journal of Biological Chemistry* **282**, 27905–27912.
192. Wang, J.F., Bown, C. and Young, L.T. (1999) Differential display PCR reveals novel targets for the mood-stabilizing drug valproate including the molecular chaperone GRP78. *Molecular Pharmacology* **55**, 521–527.
193. Bown, C.D., Wang, J.F. and Young, L.T. (2000) Increased expression of endoplasmic reticulum stress proteins following chronic valproate treatment of rat C6 glioma cells. *Neuropharmacology* **39**, 2162–2169.
194. Chen, B., Wang, J.F. and Young, L.T. (2000) Chronic valproate treatment increases expression of endoplasmic reticulum stress proteins in the rat cerebral cortex and hippocampus. *Biological Psychiatry* **48**, 658–664.
195. Shao, L., Sun, X., Xu, L. *et al.* (2006) Mood stabilizing drug lithium increases expression of endoplasmic reticulum stress proteins in primary cultured rat cerebral cortical cells. *Life Sciences* **78**, 1317–1323.
196. Wang, J.F., Azzam, J.E. and Young, L.T. (2003) Valproate inhibits oxidative damage to lipid and protein in primary cultured rat cerebrocortical cells. *Neuroscience* **116**, 485–489.
197. Shao, L., Young, L.T. and Wang, J.F. (2005) Chronic treatment with mood stabilizers lithium and valproate prevents excitotoxicity by inhibiting oxidative stress in rat cerebral cortical cells. *Biological Psychiatry* **58**, 879–884.
198. Cui, J., Shao, L., Young, L.T. *et al.* (2007) Role of glutathione in neuroprotective effects of mood stabilizing drugs lithium and valproate. *Neuroscience* **144**, 1447–1453.
199. Kim, T.Y., Bang, Y.J. and Robertson, K.D. (2006) Histone deacetylase inhibitors for cancer therapy. *Epigenetics* **1**, 14–23.
200. Mayo, M.W., Denlinger, C.E., Broad, R.M. *et al.* (2003) Ineffectiveness of histone deacetylase inhibitors to induce apoptosis involves the transcriptional activation of NF-kappa B through the Akt pathway. *Journal of Biological Chemistry* **278**, 18980–18989.
201. Liu, Y., Denlinger, C.E., Rundall, B.K. *et al.* (2006) Suberoylanilide hydroxamic acid induces Akt-mediated phosphorylation of p300, which promotes acetylation and transcriptional activation of RelA/p65. *Journal of Biological Chemistry* **281**, 31359–31368.
202. Salminen, A., Tapiola, T., Korhonen, P. *et al.* (1998) Neuronal apoptosis induced by histone deacetylase inhibitors. *Brain Research Molecular Brain Research* **61**, 203–206.
203. Suuronen, T., Huuskonen, J., Pihlaja, R. *et al.* (2003) Regulation of microglial inflammatory response by histone deacetylase inhibitors. *Journal of Neurochemistry* **87**, 407–416.
204. Adam, E., Quivy, V., Bex, F. *et al.* (2003) Potentiation of tumor necrosis factor-induced NF-kappa B activation by deacetylase inhibitors is associated with a delayed cytoplasmic reappearance of I kappa B alpha. *Molecular & Cellular Biology* **23**, 6200–6209.
205. Horion, J., Gloire, G., El Mjiyad, N. *et al.* (2007) Histone deacetylase inhibitor trichostatin A sustains sodium pervanadate-induced NF-kappaB activation by delaying IkappaBalpha mRNA resynthesis: comparison with tumor necrosis factor alpha. *Journal of Biological Chemistry* **282**, 15383–15393.

206. Kim, Y.K., Lee, E.K., Kang, J.K. *et al.* (2006) Activation of NF-kappaB by HDAC inhibitor apicidin through Sp1-dependent de novo protein synthesis: its implication for resistance to apoptosis. *Cell Death Differentiation* **13**, 2033–2041.
207. Teckman, J.H. (2004) Lack of effect of oral 4-phenylbutyrate on serum alpha-1-antitrypsin in patients with alpha-1-antitrypsin deficiency: a preliminary study. *Journal of Pediatric Gastroenterology and Nutrition* **39**, 34–37.
208. Nambiar, M.P., Warke, V.G., Fisher, C.U. *et al.* (2002) Effect of trichostatin A on human T cells resembles signaling abnormalities in T cells of patients with systemic lupus erythematosus: a new mechanism for TCR zeta chain deficiency and abnormal signaling. *Journal of Cellular Biochemistry* **85**, 459–469.
209. Mercuri, E., Bertini, E., Messina, S. *et al.* (2007) Randomized, double-blind, placebo-controlled trial of phenylbutyrate in spinal muscular atrophy. *Neurology* **68**, 51–55.
210. Lezin, A., Gillet, N., Olindo, S. *et al.* (2007) Histone deacetylase mediated transcriptional activation reduces proviral loads in HTLV-1 associated myelopathy/tropical spastic paraparesis patients. *Blood* **110**, 3722–3728.
211. Kovacs, J.J., Murphy, P.J., Gaillard, S. *et al.* (2005) HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor. *Molecular Cell* **18**, 601–607.
212. Boyault, C., Gilquin, B., Zhang, Y. *et al.* (2006) HDAC6-p97/VCP controlled polyubiquitin chain turnover. *EMBO Journal* **25**, 3357–3366.
213. Boyault, C., Zhang, Y., Fritah, S. *et al.* (2007) HDAC6 controls major cell response pathways to cytotoxic accumulation of protein aggregates. *Genes & Development* **21**, 2172–2181.
214. Gao, Y.S., Hubbert, C.C., Lu, J. *et al.* (2007) Histone deacetylase 6 regulates growth factor-induced actin remodeling and endocytosis. *Molecular & Cellular Biology* **27**, 8637–8647.
215. Fiskus, W., Ren, Y., Mohapatra, A. *et al.* (2007) Hydroxamic acid analogue histone deacetylase inhibitors attenuate estrogen receptor-alpha levels and transcriptional activity: a result of hyperacetylation and inhibition of chaperone function of heat shock protein 90. *Clinical Cancer Research* **13**, 4882–4890.
216. Wang, Y., Wang, S.Y., Zhang, X.H. *et al.* (2007) FK228 inhibits Hsp90 chaperone function in K562 cells via hyperacetylation of Hsp70. *Biochemical & Biophysical Research Communications* **356**, 998–1003.
217. Scroggins, B.T., Robzyk, K., Wang, D. *et al.* (2007) An acetylation site in the middle domain of Hsp90 regulates chaperone function. *Molecular Cell* **25**, 151–159.
218. Edwards, A., Li, J., Atadja, P. *et al.* (2007) Effect of the histone deacetylase inhibitor LBH589 against epidermal growth factor receptor-dependent human lung cancer cells. *Molecular and Cancer Therapeutics* **6**, 2515–2524.
219. Bali, P., Pranpat, M., Bradner, J. *et al.* (2005) Inhibition of histone deacetylase 6 acetylates and disrupts the chaperone function of heat shock protein 90: a novel basis for antileukemia activity of histone deacetylase inhibitors. *Journal of Biological Chemistry* **280**, 26729–26734.
220. Kong, X., Lin, Z., Liang, D. *et al.* (2006) Histone deacetylase inhibitors induce VHL and ubiquitin-independent proteasomal degradation of hypoxia-inducible factor 1alpha. *Molecular & Cellular Biology* **26**, 2019–2028.
221. Johnson, C.A., White, D.A., Lavender, J.S. *et al.* (2002) Human class I histone deacetylase complexes show enhanced catalytic activity in the presence of ATP and co-immunoprecipitate with the ATP-dependent chaperone protein Hsp70. *Journal of Biological Chemistry* **277**, 9590–9597.
222. Liu, X.L., Done, S.C., Yan, K. *et al.* (2004) Defective trafficking of nephrin missense mutants rescued by a chemical chaperone. *Journal of the American Society of Nephrology* **15**, 1731–1738.
223. Yam, G.H., Roth, J. and Zuber, C. (2007) 4-Phenylbutyrate rescues trafficking incompetent mutant alpha-galactosidase A without restoring its functionality. *Biochemical & Biophysical Research Communications* **360**, 375–380.

224. Datta, R., Waheed, A., Shah, G.N. *et al.* (2007) Signal sequence mutation in autosomal dominant form of hypoparathyroidism induces apoptosis that is corrected by a chemical chaperone. *Proceedings of the National Academy of Sciences USA* **104**, 19989–19994.
225. Watanabe, N. and Lam, E. (2007) BAX inhibitor-1 modulates endoplasmic reticulum stress-mediated programmed cell death in Arabidopsis. *Journal of Biological Chemistry*. **283**, 3200–3210.
226. Zeitlin, P.L., Diener-West, M., Rubenstein, R.C. *et al.* (2002) Evidence of CFTR function in cystic fibrosis after systemic administration of 4-phenylbutyrate. *Molecular Therapeutics* **6**, 119–126.
227. Singh, O.V., Vij, N., Mogayzel, P.J. Jr. *et al.* (2006) Pharmacoproteomics of 4-phenylbutyrate-treated IB3-1 cystic fibrosis bronchial epithelial cells. *Journal of Proteome Research* **5**, 562–571.
228. Rubenstein, R.C. and Zeitlin, P.L. (1998) A pilot clinical trial of oral sodium 4-phenylbutyrate (Buphenyl) in deltaF508-homozygous cystic fibrosis patients: partial restoration of nasal epithelial CFTR function. *American Journal of Respiratory and Critical Care Medicine* **157**, 484–490.
229. Rubenstein, R.C. and Lyons, B.M. (2001) Sodium 4-phenylbutyrate downregulates HSC70 expression by facilitating mRNA degradation. *American Journal of Physiology Lung Cellular and Molecular, Physiology* **281**, L43–L51.
230. Rubenstein, R.C., Egan, M.E. and Zeitlin, P.L. (1997) In vitro pharmacologic restoration of CFTR-mediated chloride transport with sodium 4-phenylbutyrate in cystic fibrosis epithelial cells containing delta F508-CFTR. *Journal of Clinical Investigation* **100**, 2457–2465.
231. Lim, M., Mckenzie, K., Floyd, A.D. *et al.* (2004) Modulation of deltaF508 cystic fibrosis transmembrane regulator trafficking and function with 4-phenylbutyrate and flavonoids. *American Journal of Respiratory Cell and Molecular Biology* **31**, 351–357.
232. Kontopoulos, E., Parvin, J.D. and Feany, M.B. (2006) Alpha-synuclein acts in the nucleus to inhibit histone acetylation and promote neurotoxicity. *Human Molecular Genetics* **15**, 3012–3023.
233. Choo-Kang, L.R. and Zeitlin, P.L. (2001) Induction of HSP70 promotes DeltaF508 CFTR trafficking. *American Journal of Physiology Lung Cellular and Molecular, Physiology* **281**, L58–L68.
234. Cheong, N., Madesh, M., Gonzales, L.W. *et al.* (2006) Functional and trafficking defects in ATP binding cassette A3 mutants associated with respiratory distress syndrome. *Journal of Biological Chemistry* **281**, 9791–9800.
235. Hayashi, H. and Sugiyama, Y. (2007) 4-phenylbutyrate enhances the cell surface expression and the transport capacity of wild-type and mutated bile salt export pumps. *Hepatology* **45**, 1506–1516.
236. Tveten, K., Holla, O.L., Ranheim, T. *et al.* (2007) 4-Phenylbutyrate restores the functionality of a misfolded mutant low-density lipoprotein receptor. *FEBS Journal* **274**, 1881–1893.
237. Kim, A.J., Shi, Y., Austin, R.C. *et al.* (2005) Valproate protects cells from ER stress-induced lipid accumulation and apoptosis by inhibiting glycogen synthase kinase-3. *Journal of Cell Science* **118**, 89–99.
238. Corson, T.W., Woo, K.K., Li, P.P. *et al.* (2004) Cell-type specific regulation of calreticulin and Bcl-2 expression by mood stabilizer drugs. *European Neuropsychopharmacology* **14**, 143–150.
239. Wang, J.F., Bown, C.D., Chen, B. *et al.* (2001) Identification of mood stabilizer-regulated genes by differential-display PCR. *International Journal Neuropsychopharmacology* **4**, 65–74.
240. Kim, H.J., Rowe, M., Ren, M. *et al.* (2007) Histone deacetylase inhibitors exhibit anti-inflammatory and neuroprotective effects in a rat permanent ischemic model of stroke: multiple mechanisms of action. *Journal of Pharmacology and Experimental Therapeutics* **321**, 892–901.
241. Ren, M., Leng, Y., Jeong, M. *et al.* (2004) Valproic acid reduces brain damage induced by transient focal cerebral ischemia in rats: potential roles of histone deacetylase inhibition and heat shock protein induction. *Journal of Neurochemistry* **89**, 1358–1367.

242. Morland, C., Boldingh, K.A., Iversen, E.G. *et al.* (2004) Valproate is neuroprotective against malonate toxicity in rat striatum: an association with augmentation of high-affinity glutamate uptake. *Journal of Cerebral blood flow and Metabolism* **24**, 1226–1234.
243. Inden, M., Kitamura, Y., Takeuchi, H. *et al.* (2007) Neurodegeneration of mouse nigrostriatal dopaminergic system induced by repeated oral administration of rotenone is prevented by 4-phenylbutyrate, a chemical chaperone. *Journal of Neurochemistry* **101**, 1491–1504.
244. Pan, T., Li, X., Xie, W. *et al.* (2005) Valproic acid-mediated Hsp70 induction and anti-apoptotic neuroprotection in SH-SY5Y cells. *FEBS Letters* **579**, 6716–6720.
245. Hiroi, T., Wei, H., Hough, C. *et al.* (2005) Protracted lithium treatment protects against the ER stress elicited by thapsigargin in rat PC12 cells: roles of intracellular calcium, GRP78 and Bcl-2. *Pharmacogenomics Journal* **5**, 102–111.
246. Bevins, R.L. and Zimmer, S.G. (2005) It's about time: scheduling alters effect of histone deacetylase inhibitors on camptothecin-treated cells. *Cancer Research* **65**, 6957–6966.
247. Spannhoff, A., Machmur, R., Heinke, R. *et al.* (2007) A novel arginine methyltransferase inhibitor with cellular activity. *Bioorganic & Medicinal Chemistry Letters* **17**, 4150–4153.
248. Spannhoff, A., Heinke, R., Bauer, I. *et al.* (2007) Target-based approach to inhibitors of histone arginine methyltransferases. *Journal of Medical Chemistry* **50**, 2319–2325.
249. Kubicek, S., O'sullivan, R.J., August, E.M. *et al.* (2007) Reversal of H3K9me2 by a small-molecule inhibitor for the G9a histone methyltransferase. *Molecular Cell* **25**, 473–481.
250. Greiner, D., Bonaldi, T., Eskeland, R. *et al.* (2005) Identification of a specific inhibitor of the histone methyltransferase SU(VAR)3-9. *Nature Chemical Biology* **1**, 143–145.
251. Cheng, D., Yadav, N., King, R.W. *et al.* (2004) Small molecule regulators of protein arginine methyltransferases. *Journal of Biological Chemistry* **279**, 23892–23899.
252. Miao, F., Li, S., Chavez, V. *et al.* (2006) Coactivator-associated arginine methyltransferase-1 enhances nuclear factor-kappaB-mediated gene transcription through methylation of histone H3 at arginine 17. *Molecular Endocrinology* **20**, 1562–1573.

Abstract

Multiple sclerosis is an autoimmune demyelinating disease associated with significant neurological diasbility in adults. In recent years, significant advances have been made in the identification of immunomodulatory or immunosuppressive therapies for this disease. Despite these new compounds, therapeutic efficacy is often limited to individual patients. The activation of the pro-inflammatory environment in the central nervous system of affected individuals results in the induction of expression of deleterious pro-inflammatory genes, and switches off expression of critical modulatory genes. Histone deacetylases are a class of enzymes which are critical regulators of gene expression. This chapter explores the evidence that these proteins are good candidate therapeutic targets for multiple sclerosis.

Key words: histone deacetylase; histone; chromatin; therapy; neuronal; endoplasmic reticulum; stress.

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