Stem Cell Biology and Regenerative Medicine

Paul J. Fairchild *Editor*

The Immunological Barriers to Regenerative Medicine

Humana Press

Stem Cell Biology and Regenerative Medicine

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Humana Press

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This book is dedicated to my wife, Jackie, whose unswerving love, support and encouragement over the past 25 years, remains a constant source of strength and inspiration in a rapidly-changing world

Preface

Few could doubt the need for regenerative medicine. While the increase in life expectancy we have witnessed throughout the developed world over the past 80 years is undoubtedly a medical success story of unprecedented magnitude, the accompanying increase in incidence of non-communicable diseases (NCDs), with a chronic or degenerative aetiology, represents a significant challenge of the twenty-first century. It is estimated, for instance, that the worldwide incidence of mortality due to NCDs will rise to 52 million per year by 2030, while deaths through infectious disease will continue to decline throughout the same period. Such changes in modern healthcare needs, have created an almost insatiable demand for new treatments capable of harnessing the properties of stem cells to replace diseased or effete cell types, or that rejuvenate tissues from within, through the activity of endogenous stem cells. And there have been numerous recent advances that represent significant steps towards the realisation of this vision. While the routine derivation of human embryonic stem cells (hESC) has made pluripotency accessible in man for the first time, the advent of induced pluripotency has paved the way for its clinical application to be tailored to the needs of the individual. Furthermore, preliminary successes in the treatment of diseases such as macular degeneration of the eye through cell replacement therapy suggest that we may at last be on the cusp of reaping the benefits of the past 15 years of research into the nascent field of regenerative medicine.

Nevertheless, fundamental challenges remain to be addressed before such developments may have any significant impact on global health. The British Government's *Forward look in regenerative medicine*, convened in September 2011, identified the immune response directed at stem cell-derived tissues to be a fundamental roadblock to progress. Although the early days of regenerative medicine were accompanied by unfounded optimism that tissues differentiated from hESC or, more recently, induced pluripotent stem cells (iPSC), might prove to be poorly immunogenic, it is now widely accepted that cell therapies pose no fewer immunological challenges than whole organ transplantation: indeed, unlike conventional transplants, the propensity for tumorigenesis of pluripotent stem

cells, suggests that long-term immune suppression is unlikely to offer a solution to rejection in this particular setting.

It is against such a backdrop that this volume offers an analysis of the scale and nature of the immunological issues facing regenerative medicine, drawing on the expertise of laboratories around the world who have taken up the challenge of applying their expertise in immunology to the vagaries of stem cell biology. In Part I, we explore the extent to which the principles of allograft rejection, learned over several decades from our experiences of whole organ transplantation, apply within the unique context of cell replacement therapy. Part II discusses various innovative ways of addressing the issues of immunogenicity, while, in Part III, we focus exclusively on the induction of immunological tolerance through a variety of novel approaches. It is our hope that this systematic analysis of the current state of the field will galvanise efforts to solve an issue which has so far remained intractable.

I am, of course, deeply indebted to all the authors for their patience and commitment to completing this project. Furthermore, there are many who have played an important part in its completion, often in subtle ways, and invariably without realising how important their contributions have been. I have, for instance, been inspired by many friends and colleagues, of which Bébhinn Ramsay, Steve Cobbold and Kathleen Nolan deserve special mention. The members of my laboratory should likewise be singled out, not only for their encouragement and the many scientific insights they have offered, but for the temporary neglect they have endured with such good humour. To this end, I would like to thank Tim Davies, Kate Silk, Alison Leishman, Naoki Ichiryu, Simon Hackett and Patty Sachamitr for their loyalty and for creating such a dynamic and enjoyable environment in which to work. It would be remiss of me not to acknowledge the enormous debt of gratitude I owe my mentors, past and present, for instilling in me their enthusiasm for science and its application to medicine. Jonathan Austyn, David Wraith, Richard Gardner and Herman Waldmann have all invested huge amounts of time and resources in me over the years, often with precious little reward, but their efforts have certainly not been overlooked! Finally, as is so often the case, it is my wife, Jackie, and my son, Richard, who deserve the greatest recognition for their ongoing support and unfaltering love and encouragement: without their sacrifice of holidays and our usual family Christmas, this volume would never have been completed!

Oxford, UK Paul J. Fairchild

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Part I
The Immunogenicity of Stem Cells

Chapter 1
Mechanisms of Immune Rejection of Stem Cell-Derived Tissues: Insights From Organ Transplantation

Eleanor M. Bolton and J. Andrew Bradley

Abstract The use of embryonic, induced pluripotent, or adult stem cells is upheld as a potentially valuable therapeutic approach for replacement or repair of diseased and damaged tissues, partly because these immature cells are considered to be non-immunogenic. It is becoming increasingly clear, however, that tissues differentiated from such stem cell sources have the potential to express immunogenic molecules and will be susceptible to a patient's immune response. This chapter draws on experience of organ and tissue transplantation and the study of transplant immunology to identify cellular and molecular mechanisms that are likely to be relevant to the rejection of stem cell-derived tissues. Pathways of cellular recognition and immune activation are described, together with effector mechanisms that may be responsible, not only for destruction of stem cell transplants, but also for regulating immune responses, thereby improving their chance of survival.

1.1 Introduction

Regenerative medicine is a research discipline whose aim is to establish regeneration, repair or replacement of diseased or damaged tissues, cells and organs, using a variety of approaches. It is anticipated that scientists will learn how to actively and specifically direct the differentiation of stem cells ex vivo toward the recreation of functioning tissues and organs that may be used for repair and

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replacement, and this chapter will consider the immunological implications of those aims. The term stem cells in this context includes pluripotent embryonic stem (ES) cells, induced pluripotent stem cells and adult stem cells, and their sources are, respectively, embryos at very early stages of development, terminally differentiated somatic cells, and stem cells found within adult, functioning tissues, and organs.

The use of stem cells for repair and replacement necessitates some form of tissue culture and manipulation of stem cells followed by their transfer either back into the original cell donor or into an unrelated individual. The transfer of unrelated cells is likely to invoke, to a variable degree, an immune response in the recipient that may culminate in rejection of the transferred tissue.

Several experimental studies have used embryonic or adult stem cells, and differentiated cells derived from these early developmental stages, to treat a range of animal models of human diseases, including heart disease, liver failure, diabetes, and neurodegenerative diseases but the possibility that such interventions may fail because they initiate immunological rejection which has often been overlooked. Many studies using tissue transplanted between outbred rodents or across species have failed to report whether immunosuppression was used. In studies involving transplantation of fetal or ES cell-derived neurological tissue to the brain, recognized to be an immunologically privileged site by virtue of an intact blood–brain barrier, there is a lack of consensus on the need for immunosuppression or a need to use immuno-incompetent recipients. Moreover, many other studies have reported failure of engraftment of fetal or ES cell-derived tissues that is best explained by tissue incompatibility and immunological rejection. To those involved in traditional cell and tissue transplantation this apparent oversight seems surprising as graft rejection has long been recognized as the major barrier to successful transplantation.

To understand the immunological challenges posed by transfer of stem cell-derived tissue, insight may be gained from transplantation of hematopoietic stem cells to treat patients with immune deficiency or blood malignancies. It is also relevant to refer to the extensive body of knowledge of tissue rejection gained from the study of organ transplantation. The historical assumption that stem cells are not immunogenic and therefore not susceptible immune-mediated rejection is now being challenged and many groups are studying the immunogenicity of stem cells and their differentiated progeny. This chapter reviews the immunological basis for rejection of tissue and organ allografts on the basis that many of the principles and lessons learned likely apply also to stem cell transplantation.

1.2 Historical Perspective

Following Landsteiner's discovery of human blood groups in 1900 and the recognition that blood group matching enabled successful blood transfusion, it was perhaps a logical progression to attempt to transplant other tissues between blood group

matched individuals, but early experimental attempts were met with consistent failure. The era of clinical organ transplantation began in the 1950s after the first successful kidney transplant was performed by Joseph Murray and colleagues between genetically identical twins in Boston in 1954 [1]. This and other kidney transplants between identical twins demonstrated clearly that organ transplantation was feasible if the immunological hurdles could be overcome and a search for effective immunosuppressive agents began. Renal transplantation became firmly established as a successful treatment for end-stage renal failure with the introduction of effective immunosuppressive drugs, notably a combination of the 6-mercaptopurine derivative, azathioprine, and corticosteroids [2, 3]. The requirement for immunosuppression was supported by the earlier work of the biologist, Peter Medawar who observed the rejection of skin grafts in burns patients and then went on to study the phenomenon systematically in rabbits. He was the first to show unequivocally that transplant rejection was a manifestation of the immune system recognizing the presence of "foreign", or "non-self" tissue, since skin grafts within the same individual were not rejected while grafts between unrelated individuals were always rejected. Moreover, a second graft from the same donor to a recipient that had rejected a first graft was rejected in accelerated fashion, but when the second graft was from a different unrelated donor it was rejected in normal tempo, demonstrating the development of specific immunological memory [4–6]. An understanding of these observations drew on earlier experimental studies of transfer of malignant tumors between different strains of inbred mice, where the survival or rejection of the transplanted cells was shown to be genetically controlled [7]. Another of Medawar's important contributions was the demonstration of neonatal immunological tolerance: neonatal inbred mice injected with lymphocytes from an unrelated inbred strain were unable, as adults, to reject a skin graft from the same donor strain as the injected cells while they rapidly rejected an unrelated skin graft [8]. Together with the pioneering observations of Medawar, a series of seminal advances over the next two decades provided the basis for current understanding of transplant rejection:

- Frank MacFarlane Burnet proposed the clonal selection theory to explain the development of self-tolerance and the inability to generate self-directed antibody responses [9];
- Gorer and Snell described the genetically determined "histocompatibility complex" antigens that were responsible for rejection of mismatched tissues [10];
- Gowans and colleagues demonstrated a key role for recirculating lymphocytes in both antibody responses to injected soluble antigens, and cell-mediated responses to skin grafts [11];
- Jacques Miller highlighted the importance of thymus-derived T lymphocytes in a range of immunological responses including skin graft rejection, which, together with the earlier observations of Bruce Glick on the role of Bursa-processed cells in antibody responses but not skin graft responses, revealed the dichotomy in the function of lymphocytes [12].

These and other important findings from the 1950s and 1960s established the paradigm of transplantation immunology and provided the basis for immunological

dogma that remains relevant today in the context of both tissue and stem cell transplantation.

1.3 Terms Commonly Used in Transplantation

Several technical terms are used to describe the type and origin of a transplant and to imply its likely outcome (Table 1.1). The early transplantation papers of Medawar and of Murray and colleagues referred to "autografts" and "homotransplants" or homografts. The term "autograft" (or autologous graft) is self explanatory, meaning a transplant of skin, bone marrow, or other tissue within the same individual, and is a term that is still in use. The term "homograft", in contrast, is not a useful term because while it refers to a transplant from one individual to another, it does not distinguish between a transplant from an unrelated donor and from a genetically identical donor. Instead, the terms "allograft" and "syngeneic graft" are used, in both clinical and experimental transplantation, to refer to transplants from non-identical donors and from genetically identical donors (e.g., identical twin), respectively. A xenograft is a transplant from one species to another. Only the terms autograft and syngeneic graft imply that the transplant will not elicit an immune response, and in all other cases of transplantation to a fully immunocompetent recipient, it may be assumed that unless effective immunosuppression is used, the transplant will invariably be rejected because of an immune response against non-self tissue. This applies as much to cellular transplants as to tissues and organ transplants because rejection is initiated by the presence of mismatched histocompatibility antigens that are expressed by virtually all nucleated cells of the body. The challenge, in the case of regenerative medicine, is to determine when, and to what extent, histocompatibility antigens are expressed by ES cells and their differentiated derivatives.

1.4 Tissue Compatibility

The immunological barriers to regenerative medicine are, in principle, the same as those for successful bone marrow, tissue, and organ transplantation. Rejection occurs because of allelic differences between transplant donor and recipient at a number of genetic loci that are included within the ABO blood group system, the major histocompatibility complex (MHC) and the minor histocompatibility (mH) antigens. ABO blood group antigens are expressed at the cell surface, not only of blood erythrocytes but also on most epithelial and endothelial cells. MHC molecules are also expressed at the cell surface, as class I and class II molecules which have variable tissue distribution reflecting their immunological function. Both ABO and MHC tissue antigens are, therefore, easily recognized by the immune system and may elicit powerful immune responses resulting in rapid graft rejection. mH antigens are allelic

Table 1.1 Terms in transplantation immunology

Term	Explanation
Allograft	Transplantation of tissue or organ between genetically dis-similar individuals
Syngeneic graft	Transplantation between genetically identical individuals
Autograft	Transplantation of tissue within one individual
Xenograft	Transplant from one species to another
Privileged site	An anatomical site, e.g., the anterior chamber of the eye, where transplanted tissue is protected from graft rejection
MHC	Major histocompatibility complex: the conserved gene region encoding highly polymorphic class I and class II cell surface molecules that present antigenic peptides to T lymphocytes
mH	Minor histocompatibility antigens: polymorphic intracellular proteins that, when presented as peptides, may contribute to immunological rejection
HLA complex	Human leukocyte antigen complex: term for the human MHC, located on chromosome 6
H-2 complex	Histocompatibility-2: term for the mouse MHC, located on chromosome 17

forms of intracellular proteins and are presented only as antigenic peptides; they are less easily recognized by the immune system but may contribute to, or in certain circumstances be responsible for, graft rejection.

1.4.1 The ABO System

Among cellular transplant procedures, blood transfusion is the most common and ABO blood group compatibility is necessary to ensure safe and successful transfusion. ABO antigens are protein–carbohydrate molecules, termed H antigen, inserted in the cell membrane of erythrocytes. The H antigen locus has three allelic forms that encode the terminal carbohydrate chain of the A antigen form, the B antigen form, or unchanged H antigen, designated O. All individuals have naturally occurring, circulating antibodies of the IgM class with specificity for the non-expressed A or B antigens, that develop during infancy as a cross-reaction response to bacteria colonizing the gastrointestinal tract and expressing similar surface antigens. Thus, blood group A individuals have circulating anti-B antibodies, blood group B individuals have circulating anti-A antibodies, blood group O individuals have both anti-A and anti-B antibodies while those who are blood group AB have no circulating antibodies against ABO antigens. Pre-existing IgM antibodies against ABO antigens rapidly bind to their target molecules on transfused blood or transplanted tissues, activating the complement cascade and the coagulation response, and thereby causing blood lysis and extensive tissue damage.

Since ABO antigens are expressed on many cell types other than erythrocytes, ensuring ABO compatibility is a prerequisite to bone marrow and organ

transplantation. It has recently been shown that ABO antigens are also expressed by both ES cells and by their in vitro-differentiated derivatives such as cardiomyocyte- and hepatocyte-like cells [13], suggesting that ABO matching will be necessary for regenerative medicine.

The Rhesus blood group antigens are another system of erythrocyte-expressed molecules that may elicit a strong but limited antibody response following transfusion of Rhesus-positive blood into a Rhesus-negative individual, but there are no pre-existing anti-Rhesus antibodies and it is not considered necessary to match for Rhesus antigens in tissue or organ transplantation.

1.4.2 The Major Histocompatibility Complex

The MHC is a system of around 200 genes located on the short arm of chromosome 6 in humans (at 6p21.1–21.3) and encoding, among others, three major classes of molecules, two of which have multiple allelic forms (Fig. 1.1 and Table 1.2). These gene products are collectively called Human Leukocyte Antigens, or HLA, because the molecules were originally known to be present on leukocytes but have since been shown to be widely expressed throughout the body [14, 15]. HLA class I and class II molecules have a key role in immune surveillance since their function is to present peptides derived from either newly generated intracellular proteins (including viral proteins) or proteins sampled from the extracellular environment, for presentation to T lymphocytes. Depending on the nature of the peptide, T cells will either be responsive or anergic. The HLA system is the most highly polymorphic gene system in the body; it includes 3 highly polymorphic class I genes whose allelic forms of α-chains combine with the non-polymorphic β2-microglobulin chain to form the heterodimeric class I molecules HLA-A, HLA-B, and HLA-C which are widely expressed in the cell membranes of most nucleated cells in the body. The HLA system also includes three pairs of polymorphic class II α- and β-chain genes whose gene products combine to form the heterodimeric HLA-DR, -DP, and -DQ class II molecules inserted in the cell membranes of specialized leukocytes collectively termed antigen presenting cells (APC), as well as endothelial cells, and certain types of epithelial cells. Their distribution is much less widespread than that of class I molecules. Expression of HLA class I and class II may be both highly upregulated and induced in the presence of pro-inflammatory cytokines, particularly interferon-γ. The HLA system also encodes other, relatively non-polymorphic class I molecules whose tissue distribution is restricted, such as HLA-E and HLA-G which function as recognition elements for cells of the innate immune system, including natural killer (NK) cells. NK cells typically kill cells that express no, or low, classical HLA class I and are facilitated to recognize absence of classical class I by the presence of non-classical class I. Thus, during pregnancy, the trophoblast does not express classical HLA class I, to protect the semi-allogeneic fetus from immune attack, but it does express high levels of HLA-G that engage with NK cell receptors and protect the

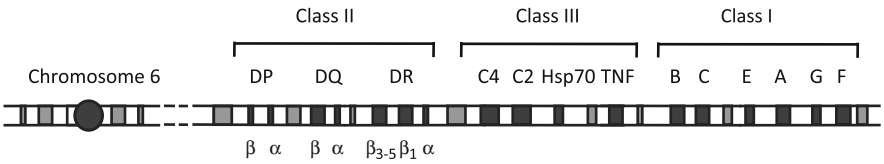

Fig. 1.1 The HLA gene complex. The HLA class I and class II membrane molecules, together with soluble inflammatory proteins (class III), are encoded by a set of genes located on the short arm of chromosome 6, at 6p21.1–21.3

Table 1.2 The HLA gene complex

HLA class	HLA locus	HLA alleles	HLA serological specificities
Class I	A	>1000	28
Class I	B	>1600	60
Class I	C	>650	10
Class II	DRα	3	24
	DRβ1	>750	
	DRβ3-5	>70	
Class II	DPα	>25	6
	DPβ	>135	
Class II	DQα	>30	9
	DQβ	>100	

The HLA class I and class II membrane molecules, together with soluble inflammatory proteins (class III), are encoded by a set of genes located on the short arm of chromosome 6. According to recent data, a total of 3296 HLA-A, -B and -C α-chain alleles encode 2520 proteins, of which 98 are recognized as distinct class I molecules by anti-HLA antibodies [18]. Similarly, 1222 α- and β-chain alleles encode 931 class II molecules of which 39 are recognized by specific antibodies

trophoblast from attack. Other relatively non-polymorphic class II genes encode various proteins involved in antigen processing and presentation, and include the proteasome component LMP genes, the class I-peptide complex assembly genes for TAP1, TAP2 and tapasin, and the class II-peptide complex assembly genes for DM and DO. HLA class III genes encode components of the complement system and certain inflammatory proteins.

As well as having a functional role in antigen presentation, HLA molecules serve as recognition elements for immune cells surveying the body. Because immune cells recognize specific peptides only when presented by APCs bearing MHC molecules identical to those expressed by the lymphocytes themselves, they are able to distinguish different peptides and different MHC molecules—a function termed MHC restriction [16]. However, they are also able to respond to non-self classical MHC molecules in a response that is unique to transplantation, in that a rejection response is initiated in an attempt to destroy the transplanted tissue. For this reason, transplantation usually requires that, where possible, donor and recipient HLA types are closely matched in order not only to minimize the amount of immunosuppression administered, but also to prevent rejection of the transplant and to reduce the risk of graft versus host disease. It is likely that HLA matching

would be advantageous for stem cell transplantation since at some point, HLA molecules will be expressed by the differentiated progeny which may then become targets of a rejection response.

1.4.3 HLA Matching

HLA matching, while desirable, is not a simple matter. There are currently more than 3000 known HLA-A, -B, and -C class I α-chain alleles and more than 1000 HLA class II α- and β-chain alleles, expressed as >2500 distinct class I molecules and >900 class II molecules, although there are only around 140 distinct epitopes recognized by individual antibodies [17, 18]. In a transplant setting, all of these distinct proteins may be antigenic since they are readily accessible to T and B lymphocyte receptors. Moreover, since they are expressed on fetal tissues and on blood cells, any potential transplant recipients that have been pregnant or had a blood transfusion may have become sensitized to non-self HLA molecules and will have generated memory T cells and possibly also circulating anti-HLA antibodies and memory B cells.

Each individual inherits their complement of two HLA alleles at each genetic locus within a section of chromosome inherited from each parent; they will express one allele each of HLA-A, HLA-B, and HLA-C classical class I molecules from each parent and one allele each of the three principal class II molecules (HLA-DR, -DP, and -DQ). Alleles are expressed co-dominantly with little or no crossover within the HLA complex (Fig. 1.2).

In the case of deceased donor kidney transplantation, the HLA tissue type of the deceased donor and all potential recipients is determined; for each donor, attempts are then made to select recipients from the transplant waiting list that are well-matched for HLA-A, -B, and -DR locus antigens. Such matching confers a survival advantage for the transplant by minimizing the risk of rejection and reducing the burden of immunosuppression. A further advantage of a kidney graft that is well matched for HLA is that, should the graft subsequently fail, it is less likely the recipient will develop anti-HLA antibodies that might rule out a second transplant. A cross match test is also performed on the selected kidney donor–recipient pair to exclude the possibility of rapid or hyperacute rejection resulting from existing circulating anti-donor HLA antibodies. For bone marrow transplantation (or hematopoietic stem cell transplantation) where the donor is not an HLA-identical sibling, HLA matching requirements are more stringent. The aim is to achieve a match at the HLA-A, -B, -C, -DR, and -DQ loci, not only to minimize the risks of rejection of the transplant but also to reduce the chance that the immune cells that constitute the transplant may themselves recognize the host as foreign and give rise to graft versus host disease.

For hematopoietic stem cell transplantation and for renal transplantation the benefits of HLA matching have long been known and remain in spite of improvements in immunosuppression (Fig. 1.3). In the case of other types of solid organ

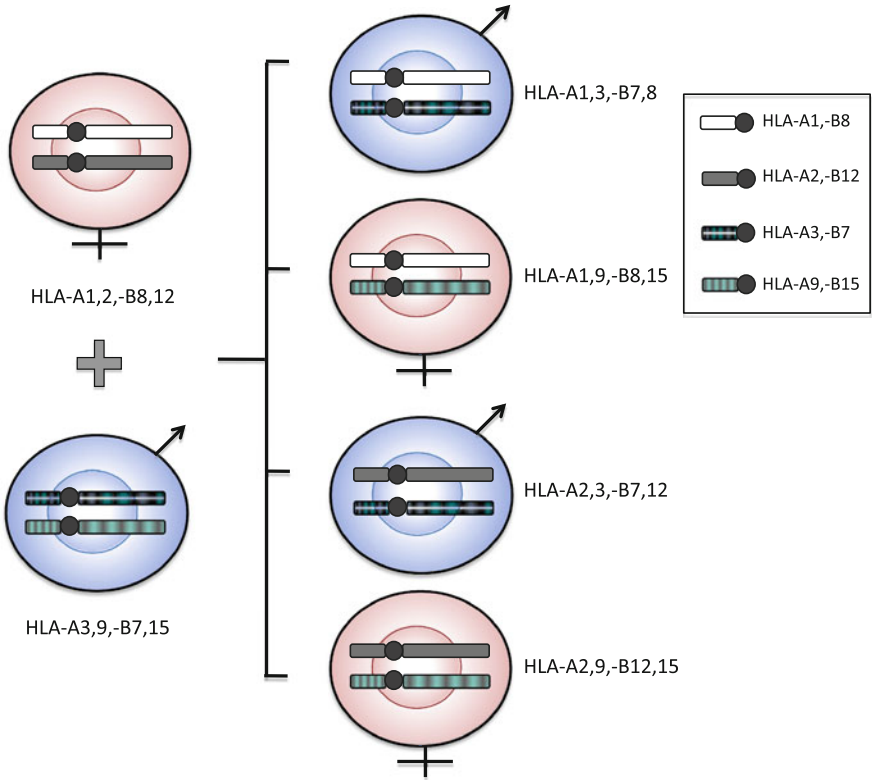

Fig. 1.2 Inheritance of HLA. An individual inherits one copy (haplotype) of the full complement of MHC genes from each parent, and expresses them co-dominantly. Chromosomal cross-over within the MHC is rare. There is a 50 % chance that an individual will have a 1-haplotype HLA match with a sibling, a 25 % chance of a 2-haplotype match, and a 25 % chance of a 2-haplotype mismatch

transplantation, including heart, lungs, and liver, HLA matching is not usually undertaken because any potential advantage of HLA matching is outweighed by the logistic difficulties of finding a well-matched organ for the smaller pool of recipients, the need to consider other factors such as size matching when allocating such organs, and the need to transplant such life-saving organs more promptly before their function is impaired by excessive cold ischemia during storage and transport.

1.4.4 Minor Histocompatibility Antigens

Minor histocompatibility antigens are protein molecules, usually with allelic variants, that are encoded by genes outside of the MHC and take the form of intracellular, rather than membrane proteins. Because of their intracellular distribution they are not

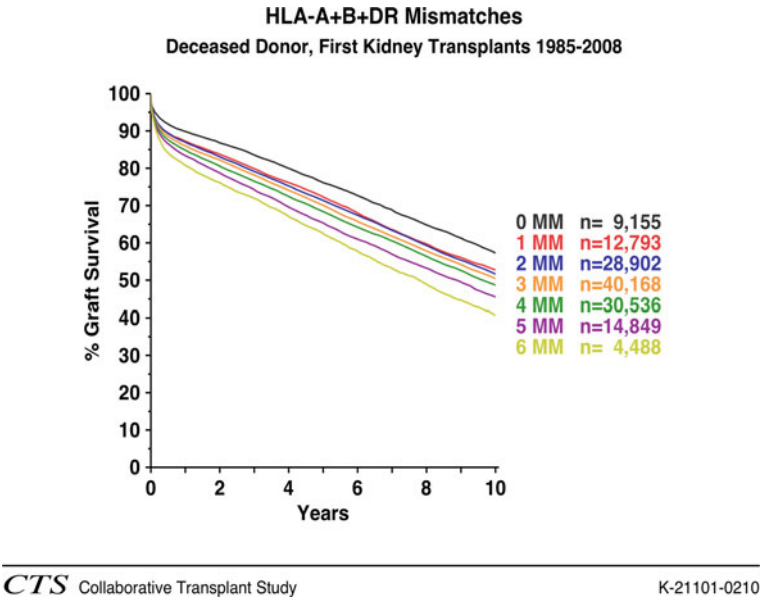

Fig. 1.3 Effect of HLA matching on outcome of renal transplants. Kaplan–Meier plot of kidney graft survival according to number of HLA mismatches (MM) between donor and recipient (where 0 MM represents a full match at each of the two HLA-A, two HLA-B, and two HLA-DR loci, and 6 MM represents expression of different alleles at each of the 6 HLA loci) demonstrating the beneficial effect of HLA matching. Data from the Collaborative Transplant Study (www.ctstransplant.org) reproduced with a kind permission from Professor Gerhard Opelz, University of Heidelberg. This color image is reproduced in grayscale; the lines of the graph are in the same order as the key, with the top line representing 0 MM and the bottom line representing 6 MM

recognized as intact proteins but rather as peptide fragments in the context of MHC. A well-known example of a mH molecule is the male-specific H-Y antigen which, in mice, is capable of causing rejection of male tissue transplanted to a female recipient [19]. An important source of genetic variation in ES cell lines generated from embryos created by nuclear transfer, is mitochondrial gene products, which provide another example of mH antigens. Characteristically, mH antigens contribute to rejection but at a slower tempo when compared with MHC antigens. Clinically, no attempt is made to match for mH antigens prior to transplantation but it is clear from HLA-matched hematopoietic stem cell transplant patients that there remains a requirement for immunosuppression to counteract rejection induced by minor antigen mismatches.

1.5 HLA Structure and Function

The discovery of MHC molecules and their genetic diversity arose from tumor transplantation experiments in mice, where it became clear that blood lymphocytes could recognize and proliferate in response to exposure to non-self MHC

molecules expressed on the cells of genetically unrelated mice. It was puzzling that a system of highly visible, highly polymorphic molecules should exist and that there was a need for their specific recognition by cells of the immune system and by antibodies. During the 1970s it was shown that the function of MHC molecules was to serve as a recognition element for responding lymphocytes, not alone but as a complex with antigenic molecules representing foreign proteins and pathogens. The paradigm of MHC restriction (described by Zinkernagel and Doherty, [16]) was developed from the finding that a clone of T lymphocytes generated by immunizing a strain A mouse with protein X would recognize and respond (by proliferating) to cells expressing strain A MHC complexed with peptide X, but not to strain B MHC complexed with peptide X, nor to strain A MHC complexed with peptide Y. The use of crystallography to reveal the structure of the HLA-A2 molecule, in a landmark paper by Björkman and colleagues in 1987, clarified both the detailed structure of HLA class I molecules and how structure defined their function [20]. The subsequent publication of the structure of class II molecules and T cell receptors (TCRs) completed the picture and provided an understanding of the basis of an immune response: lymphocyte interactions with peptide-MHC complexes [21, 22].

1.5.1 HLA Structure

HLA class I molecules consist of two polypeptide chains of unequal size (Fig. 1.4). The extracellular region of the heavy chain, or α chain has approximately 300 amino acids arranged in three "domains", and includes a transmembrane region as well as a short intracytoplasmic tail. The heavy chain is bound non-covalently to the invariant light chain (*β*-microglobulin) that does not have a transmembrane region. The two distal α1 and α2 domains form the antigen-binding part of the class I molecule, while the membrane-proximal α3 domain has an invariant region that binds weakly to the CD8α molecule during interaction with $CD8^+$ T cells. The α1 and α2 domains each have an area of *β*-pleated sheet surmounted by an α-helical region which together form a peptide binding cleft into which a peptide of around 9 amino acids is inserted. The structure of these two domains is such that the α-helices "present" antigenic peptide for recognition by the antigen-binding regions on the α and *β* chains of the TCR.

HLA class II molecules have been shown, by crystallography studies, to have a similar overall structure to that of class I molecules. Class II molecules have two similar sized, non-covalently bound polypeptide chains, termed α and *β*, each consisting of two extracellular domains, a transmembrane region and a cytoplasmic tail. The distal domain of each chain (the α1 and *β*1 domains) together form a structure that closely resembles the α1 and α2 domain structure of the class I molecule: each of the α1 and *β*1 domains has a region of *β*-pleated sheet surmounted by an α-helical region which together form a peptide binding cleft. The cleft of class II molecules is a more open-ended structure and, typically,

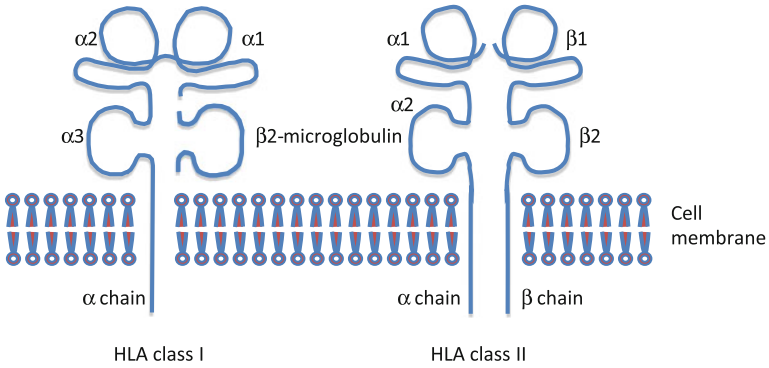

Fig. 1.4 Diagram of the structure of HLA class I and class II molecules. In both molecules, the membrane-proximal, immunoglobulin-like domains have relatively conserved amino acid sequences and provide a site for binding of the accessory CD8 and CD4 molecules, respectively, to strengthen the interaction between T cell and APC. The two distal domains of each molecule have highly polymorphic regions to ensure presentation of a wide range of peptides to the TCR

peptides of around 13 amino acids are presented to the TCR, although peptides can be much longer and have a looped conformation in the cleft. The $\alpha 2$ and $\beta 2$ domains are relatively non-polymorphic and a region of hydrophobic amino acids on each domain where they are closely approximated forms a crevice that is the site for interaction with the CD4 molecule on T lymphocytes.

1.5.2 HLA Function

Extensive gene polymorphism is critical to the function of MHC (or HLA) molecules. Both the β-pleated sheets and the α-helices of both class I and class II molecules have highly polymorphic regions. The resulting variability in amino acid sequences permits diversity of both the peptide binding elements and of the recognition elements presented to the TCR, thereby ensuring that any pathogen encountered is accessible to the immune system. This clearly gives a survival advantage to the species or strain with greatest diversity but is not helpful for regenerative medicine and transplantation.

HLA class I and class II molecules have different cellular distribution which reflects their function. Class I molecules are widely expressed on most nucleated cells throughout the body and their function is to protect the individual from intracellular pathogens such as viruses that replicate by using the host cell replication machinery. Intracellular proteins and peptides are normally packaged for presentation by class I molecules at the cell surface where they can be sampled by $CD8^+$ cytotoxic T cells (Fig. 1.5); as new viral particles are produced their peptides are transported by class I molecules to the cell surface where they are recognized by cytotoxic T cells as foreign, and they respond by killing the infected

target cells. In contrast, the function of class II molecules is to present peptides derived from extracellular proteins and pathogens. Extracellular material is sampled by phagocytosis, or macropinocytosis, or in the case of B lymphocytes, by receptor-mediated endocytosis using the specific B cell receptor or surface immunoglobulin (Fig. 1.5). The resulting membrane-bound vesicles containing potentially dangerous material become increasingly acidic, a process which helps to break down the contents. Endosomes then fuse with lysosomes that break down the contents further into peptides, which are then able to bind to class II molecules and the complex is delivered to the cell surface for presentation to CD4 T lymphocytes. Only specialized APCs, including dendritic cells, macrophages, and B lymphocytes are able to process extracellular material in this way and therefore they are the principal cell types that express class II molecules.

1.6 Induction of the Innate and Adaptive Immune Responses

Expression of MHC molecules is integral to the good health and survival of the species and it may, therefore, be assumed that at some stage in its life cycle, every nucleated cell will express class I molecules, if not class II molecules as well. T cell recognition of MHC-peptide is the first step toward raising an immune response against a potentially dangerous pathogen, and following transplantation, T cell recognition of non-self MHC (expressed on the donor tissue) initiates a rejection response.

The first stage in an adaptive immune response is recognition by $CD4^+$ T cells of an HLA class II-peptide complex. Unless this is a transplant situation, the $CD4^+$ T cell will recognize HLA class II as self, and the peptide as either derived from self-protein, in which case the T cell will normally be tolerant of it, or as foreign peptide, in which case the T cell will become activated. The $CD4^+$ T cell then functions as a helper cell and secretes cytokines that potentially co-ordinate the activation of the entire repertoire of the immune system, termed the adaptive immune response (or acquired immunity). Naïve $CD8^+$ T cells and B lymphocytes differentiate into cytotoxic cells and plasma cells, respectively, but only if they first receive help from activated $CD4^+$ T cells. At the same time, the innate immune response is activated by a range of different stimuli and this system contributes to adaptive immunity [23]. As the response progresses, the adaptive immune system develops specific memory of that particular antigen and if the antigen is encountered at a future date, the resulting immune response will draw on its immunological memory and will respond both more quickly and with greater magnitude. The characteristic features of adaptive immunity are specificity and memory, which are largely absent from innate immunity.

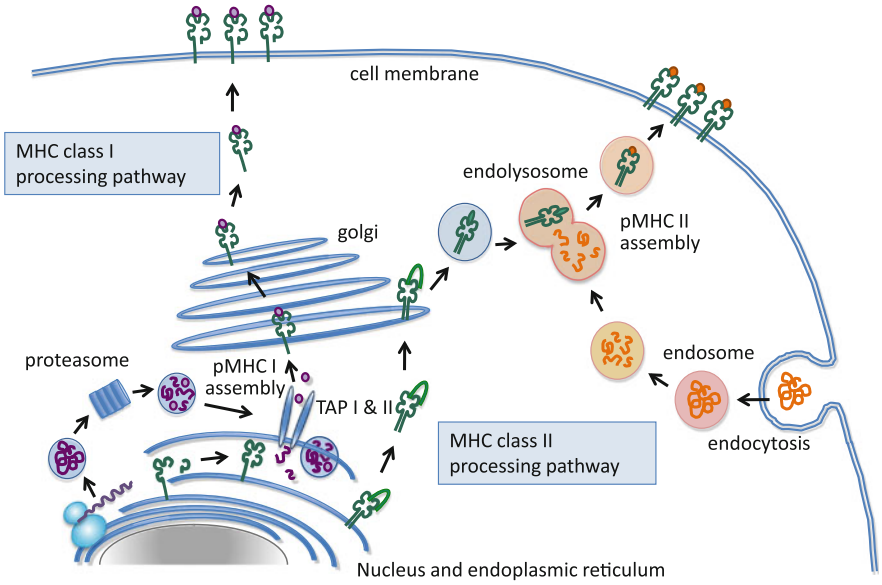

Fig. 1.5 Pathways of antigen processing and presentation. Newly synthesized endogenous proteins (including viral components) are processed and presented by MHC class I molecules, while extracellular proteins are taken up by endocytosis and processed and presented primarily by MHC class II molecules. New protein synthesis occurs when mRNA attaches to a ribosome and the ribosome attaches to ER. In order to maintain a healthy turnover of proteins, ubiquitin-tagged proteins (both normal and mis-folded) are degraded by proteasomes in the cytosol, and further degraded to peptides in the heterodimeric TAP (Transporter associated with antigen processing) molecules located in the ER. MHC class I molecules are simultaneously synthesized at the ER and the correct folding of the heavy chain with β2-microglobulin is stabilized by calnexin. Calnexin is replaced by the class I chaperone proteins, calreticulin, and tapasin, that mediate assembly of the class I molecule with peptide emerging from the TAP molecule. The free MHC class I-peptide complex is transported via the Golgi apparatus to the cell surface where it is embedded in the cell membrane for presentation to $CD8^+$ T cells. MHC class II molecules are synthesised at the ER where the two chains are complexed with the "invariant chain". This complex passes through the Golgi apparatus to be released in lysosomal vacuoles within the cytosol, where the invariant chain is shortened to become the CLIP (Class II associated invariant chain peptide). At the same time, extracellular proteins taken up by endocytosis and enclosed within endosomes are degraded to peptides as the vacuolar pH is reduced. Endosomes and lysosomes eventually fuse and the class II-region HLA-DM molecule facilitates exchange of the CLIP for antigenic peptide to form the MHC class II-peptide complex (a process that may be inhibited, instead, by the HLA-DO molecule). This complex is transported and inserted within the cell membrane for presentation to $CD4^+$ T cells. Two additional pathways, termed autophagy and cross-presentation, enable presentation of endogenous (viral) proteins by MHC class II and exogenous proteins (engulfed, virus-infected dead cells, for example) by MHC class I molecules. These are strictly regulated pathways but are important for provision of help initially for maturation of anti-viral cytotoxic T cells when viruses infect stromal cells that are not professional APCs and therefore lack co-stimulatory molecules. There is evidence that cross-presentation may occur in processing and presentation of alloantigens following transplantation [89]

1.6.1 Innate Immunity

The process of transplantation is inevitably associated with tissue damage through surgery, exposure to potentially infectious agents, and ischemia (cessation of blood supply) followed by reperfusion, all of which are powerful triggers of innate immunity [24] (Fig. 1.6). The production of free radicals, or reactive oxygen species (ROS), is characteristic of ischemic tissue damage followed by reperfusion and is a potent inducer of apoptosis via induction of caspases such as caspase 3. Production of ROS may also be induced by factors in the transplant recipient, including hypertension, hyperlipidemia, viral infections, and immunosuppressive drug toxicity [23]. Tissue damage also induces the production of heat shock proteins and other cellular proteins whose function is to scavenge harmful molecules like ROS. These scavenger proteins express simple repeating molecular patterns termed *damage-associated molecular patterns* or DAMPs that are recognized by receptors termed *Toll-like receptors* (TLRs) expressed by macrophages, neutrophils, NK cells, and dendritic cells [25, 26]. Another important trigger of innate immunity is the introduction of infectious agents where components of bacterial cell walls termed *pattern-associated molecular patterns* (or PAMPs), and single-stranded viral RNA nucleoside components, are recognized by additional members of the family of TLRs expressed by non-specific inflammatory immune cells [27, 28]. The resulting inflammatory environment activates dendritic cells to initiate antigen uptake, processing and presentation, recruits more inflammatory cells via induction of chemokines that regulate cell migration, enhances vascular permeability to encourage drainage of extracellular fluid and free soluble antigen to the draining lymph nodes, and also assists in upregulation of HLA class I and II expression. There is a considerable redundancy of TLR signaling and adaptor protein molecules in the innate response, and they play multiple roles in alloimmunity as illustrated, for example, by studies in TLR-knockout mice demonstrating a critical contribution of the innate response to acute allograft rejection, and maintenance of tolerance (abrogated by administration of TLR ligands) [29–31].

1.6.2 Adaptive Immunity

T cells residing in lymph nodes draining the site of an organ transplant encounter activated donor dendritic cells that migrate out of the transplant when blood circulation is restored, as well as recipient dendritic cells that are able to process and present donor material, such as necrotic cells, shed from the transplant. At this point, the adaptive immune response is initiated as naïve T cells engage with HLA molecules expressed by dendritic cells. Migration of T cells and dendritic cells is critical to the development of adaptive immunity and is mediated by chemokine/chemokine receptor interaction and by integrins. Chemokines are small proteins

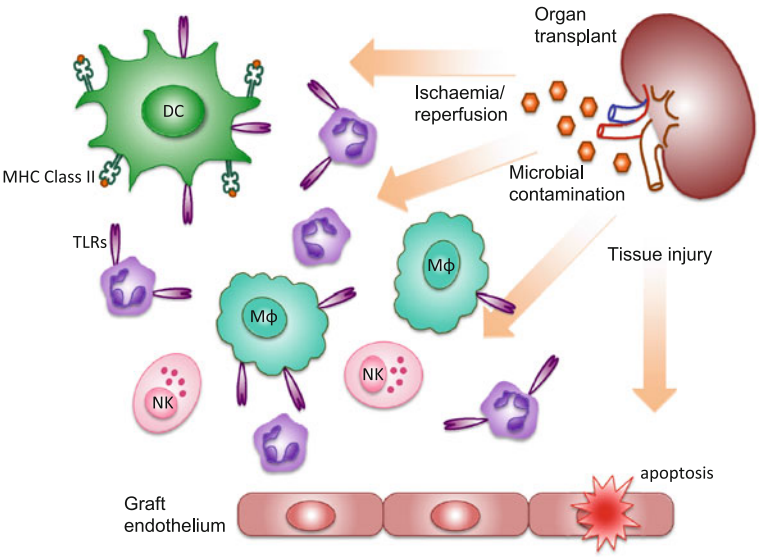

Fig. 1.6 The innate immune response. The process of organ transplantation introduces several triggers of innate immunity, including trauma and tissue damage, ischemia and reperfusion injury, and microbial contamination (viral particles are represented in the figure). Cells of the innate immune system, including dendritic cells, neutrophils, NK cells, and macrophages, express TLRs that engage with a range of molecules such as heat shock proteins released during ischemia/reperfusion injury and donor tissue injury, as well as with pathogen-associated molecular patterns (PAMPs) expressed by microbial contaminants. ROS induced by ischemia/reperfusion injury cause endothelial cell activation and apoptosis, while TLR signaling induces secretion of inflammatory proteins and activates dendritic cells, thereby initiating a link with the adaptive immune (rejection) response

with broad overall similarity that are categorized according to the structural arrangements of cysteine residues that assist in their tertiary folding [32]. Their function is to direct cell migration and they are key mediators of a range of responses involving migration, including immunity, inflammation, homeostasis, wound healing, and angiogenesis. They are produced by a wide range of cell types, including leukocytes and parenchymal cells, following a stimulus, such as viral infection, oncogenesis, and ischemia. Naive T lymphocytes express a set of chemokine receptors (particularly CCR7) that are responsive to concentration gradients of certain chemokines (particularly CCL21) produced by activated macrophages and dendritic cells within secondary lymphoid tissues. This response initiates interaction between naive T cells and APCs, and following antigen recognition, T cells express different chemokine receptors that assist their migration to appropriate areas of the lymphoid tissue where they mature, proliferate, and interact with B lymphocytes that also mature into antibody-producing cells. The contribution of organized secondary lymphoid tissue is critical to the development of an effective rejection response, as demonstrated by the diminished ability of mice lacking secondary lymphoid tissue to acutely reject an organ allograft [33]. In the presence

of an ongoing, chronic rejection response (and in chronic inflammatory autoimmune disease), however, there is evidence for lymphoid neogenesis as accumulations of lymphocytes and dendritic cells may form organized tertiary lymphoid structures within the transplant (or inflamed tissue) that may contribute to a persistent immune response [34, 35].

Lymphocytes that have encountered alloantigen presented by dendritic cells in organized lymphoid tissue are then able to respond in a chemotactic manner to chemokines produced at a distant site of inflammation, or immune stimulus. Their passage through endothelial layers into parenchymal tissue is assisted by a chemokine-induced conformational change in different integrins or adhesion molecules, expressed by both lymphocytes and endothelial cells, which permits their interaction and thereby regulates rolling of lymphocytes along endothelium, arrest, adherence, and transmigration both between and through endothelial cells to the extracellular matrix of parenchymal tissue (Fig. 1.7). Several studies have examined the contribution of chemokines and their receptors to allograft rejection and it is clear that certain interactions play a significant role under defined conditions in the outcome of experimental and clinical transplants, but also that there is considerable functional overlap between these molecules [36].

1.6.3 Natural Cytotoxicity

An important component of innate immunity is contributed by NK cells that are triggered to lyse cells expressing no, or low levels of, classical MHC class I antigens, irrespective of whether they are of autologous or allogeneic origin [37]. They have potent cytolytic activity and secrete a range of cytokines, thereby playing an important role in inflammation and regulation of adaptive immunity. NK cell activity is highly regulated via two sets of receptors:

- inhibitory killer cell immunoglobulin-like receptors (KIRs) in humans and Ly49 receptors in mice, that are induced by immuno-receptor tyrosine-based inhibitory motifs (ITIMs) on classical MHC class I molecules, and NKG2A/CD94 receptors that recognize certain non-classical MHC class I molecules (e.g., HLA-E);
- activatory or "natural cytotoxicity" receptors, including (among others) NKG2D, a transmembrane, lectin-like receptor that recognizes numerous ligands all allied to MHC class I proteins, and including MHC class I chain-related protein A (MICA) and B (MICB) which are expressed as a result of target cell stress.

NK cell activity is induced by cells that are transformed during oncogenesis and viral infection, both of which result in upregulation of NKG2D receptors and downregulation of MHC class I expression. NK cells perform an important function in hematopoietic stem cell transplantation for leukemia therapy following recipient bone marrow ablation, since donor NK cells are able to target any remaining leukemic cells, a response known as the graft versus leukemia effect [38].

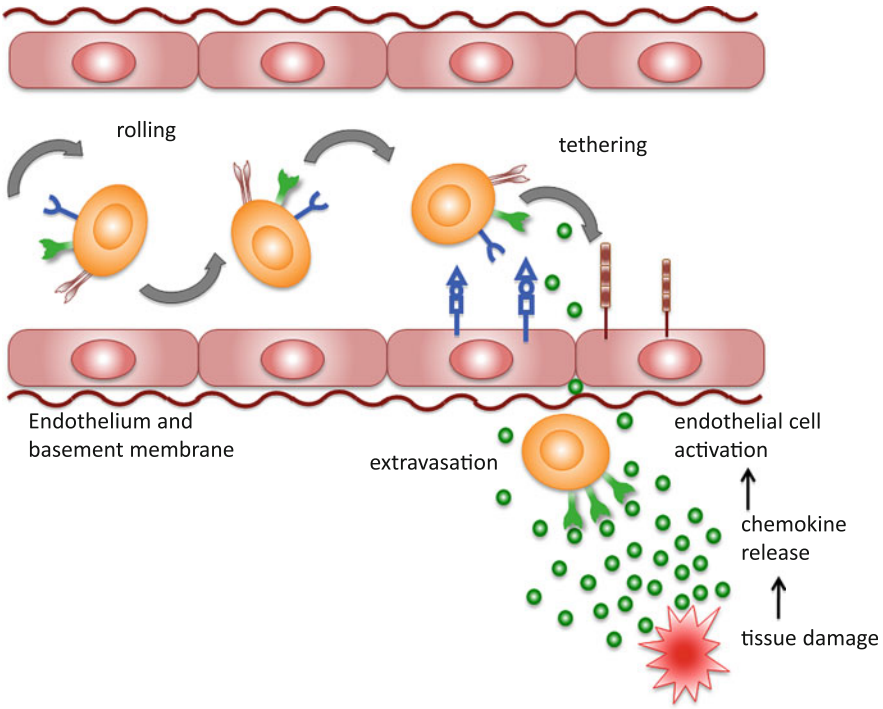

Fig. 1.7 Leukocyte adhesion and extravasation. When leukocytes encounter an area of inflammation, reduced blood flow caused by blood vessel dilation enables their interaction with endothelium via adhesion molecules on both leukocytes and endothelial cells that are induced by inflammatory cytokines such as TNF-α. Initially, selectins allow rolling adhesion to the endothelial layer, which then facilitates a tighter interaction via integrins, causing arrest of the leukocytes. They are then able to respond, via chemokine receptors, to chemokines secreted by inflammatory cells at the site of tissue injury, and use ICAM-1 and CD31 on endothelial cells to migrate along a chemokine gradient through the endothelial cell layer to the inflammatory site

In contrast, however, it has long been known that normal lymphocytes injected intravenously into an allogeneic host are rapidly destroyed and cannot be detected after 24–48 h, a time frame that is too rapid to be explained by adaptive immunity. This phenomenon is termed allogeneic lymphocyte cytotoxicity and is known to be associated with NK cell frequency, but is not compatible with the "missing self" hypothesis of NK cell targets since MHC class I is expressed on leukocyte membranes [39]. Recent research demonstrates that NK cells have some degree of allorecognition and that target cells are susceptible to natural cytotoxicity if their MHC alleles are incompatible with host NK inhibitory KIRs. This may have important implications for stem cell transplantation whether for regenerative purposes or for treatment of leukemia [38].

1.7 T Cell Recognition of Transplanted Tissues

ES cell-derived tissues differ from normally transplanted tissues, at least in the early stages of differentiation, in that they are not vascularized and do not contain dendritic cells. If they are successfully transplanted to a host, neovascularization will occur and the stem cell-derived tissue will become populated by a vascular network and by dendritic cells of host origin. It may appear, therefore, that there is no mechanism for the host immune system to recognize the stem cell-derived transplant, whether syngeneic or allogeneic, since there are no dendritic cells to migrate to the draining lymph nodes and alert host $CD4^+$ T cells. Moreover, there are no donor HLA class II-positive structures in the stem cell-derived tissue for recognition by host $CD4^+$ T helper cells. Nevertheless, stem cell-derived tissues express low levels of HLA class I, and have the potential to upregulate both class I and class II antigen expression in an inflammatory environment, which would undoubtedly trigger a rejection response in an HLA-mismatched host.

T cells recognize alloantigens (HLA antigens expressed by the HLA-mismatched donor) by two distinct pathways, termed the direct and indirect pathways of allo recognition (Fig. 1.8). The direct pathway of allorecognition is unique to transplantation: host T cells recognize intact allogeneic HLA-peptide complexes on the surface of *donor* APCs from the graft. Indirect allorecognition is not dependent on *donor* APCs since host T cells recognize donor HLA in the form of processed peptide presented in the peptide binding cleft of *recipient* APCs. This second pathway is analogous to the normal T cell response to foreign proteins and pathogens, and occurs when material is shed from the graft and is picked up, processed, and presented by host APCs.

In a normal (indirect pathway) immune response to protein antigen, only a very small percentage of circulating T cells is able to recognize and respond to any given HLA-peptide complex; the frequency of such cells is in the order of one per tens of thousands of cells. The frequency of T cells responding to transplantation antigens, however, is very high (and may be 1–5 % of total T cells) and, moreover, the response is much more vigorous because T cells recognizing alloantigen via the direct pathway have access to many more target HLA molecules [40, 41]. There are two principal hypotheses to explain the high frequency of alloreactive T cells, termed the multiple binary complex hypothesis [42] and the high determinant density hypothesis [43], both of which were proposed before crystallography gave some insight into precisely how the TCR recognizes a MHC-peptide complex (Fig. 1.9).

The first hypothesis proposes that an alloantigen is processed to produce several different antigenic peptides, each of which forms a complex with MHC and can stimulate a different clone of T cells to respond. The second hypothesis proposes that donor MHC-responsive T cell clones recognize the MHC part of the peptide-MHC complex with highest priority, so that essentially all MHC molecules expressed on an APC are recognized as foreign, irrespective of the nature of the peptide bound within their peptide binding groove. This is in marked contrast to a normal immune response to a protein antigen where only a relatively small percentage of MHC molecules display the antigenic peptide and most express non-antigenic self peptide. These

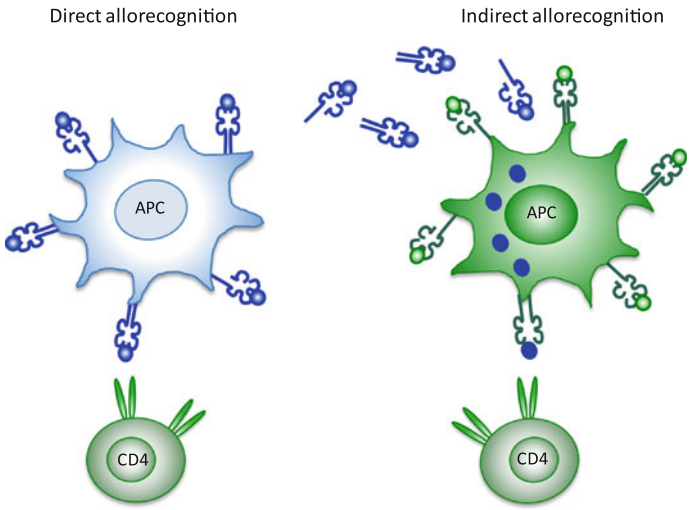

Fig. 1.8 Direct and indirect pathways of allorecognition. Transplantation is unique in that both donor and recipient dendritic cells may function as APCs. In direct allorecognition, recipient T lymphocytes recognize (by cross-reactivity) and respond to intact, non-self class I and class II HLA molecules expressed on donor-derived APCs. In indirect allorecognition, recipient $CD4^+$ T lymphocytes recognize and respond to donor HLA molecules that have been taken up and processed by recipient APCs, and presented as peptide fragments in the peptide binding cleft of recipient HLA class II molecules

explanations appear to contradict the immunological dogma of MHC restriction, whereby T cells only recognize and respond to specific peptide presented by *self* MHC, and instead depend on the concept that alloreactive T cells respond to allo-MHC-peptide complexes with lower affinity and by a cross-reactive interaction, such that they do not distinguish between certain similar but different MHC molecules, or certain similar but different peptides, or both. This conundrum remains unresolved, but irrespective of mechanism, alloantigens are more likely to provoke vigorous T cell responses than normal protein antigens because alloreactive T cells are present at high frequency.

The relative contribution of the direct and indirect allorecognition pathways is not known with certainty, but for organ transplantation at least, it is thought that donor dendritic cells activated by ischemic injury and migrating out of the graft following reperfusion are potent inducers of an acute rejection response via direct allorecognition. In the weeks and months following transplantation, donor dendritic cells are replaced with recipient dendritic cells, which are able to maintain the rejection response via the indirect pathway of allorecognition.

A recently-proposed third pathway of allorecognition has the potential to combine these two pathways and has been termed the semi-direct pathway [44]. It is dependent upon the well-recognized phenomenon of contact-dependent membrane sharing between dendritic cells and other cell types [45, 46]. APCs may therefore be able to present both self MHC complexed with allogeneic peptide, as well as intact allogeneic MHC which may enhance recipient T cell activation.

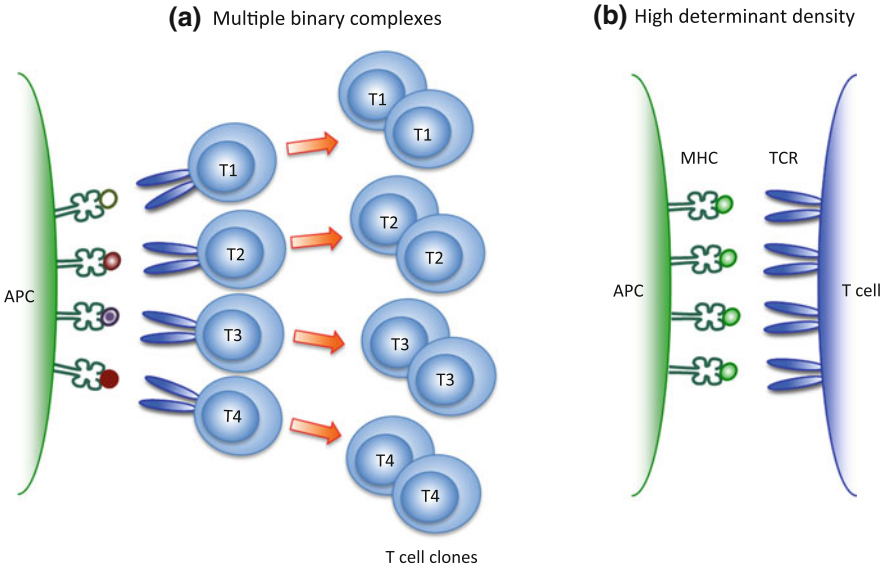

Fig. 1.9 Two hypotheses accounting for the high frequency of alloreactive T cells. **a** The multiple binary complex hypothesis proposes that T cells preferentially recognizing the peptide part of the MHC-peptide complex are able to respond by cross reaction to many donor MHC-peptide complexes presenting different endogenous peptides. **b** The high determinant density hypothesis proposes that T cells preferentially recognizing the MHC part of the MHC-peptide complex encounter alloantigen (donor MHC) that is expressed at high frequency on donor APCs, providing a strong activatory stimulus to the T cells

Once allorecognition has occurred, via either the direct or indirect pathway, and a rejection response ensues, T and B cells develop a memory phenotype and are no longer dependent on $CD4^+$ T cell help: they circulate through the transplant and are able to recognize target alloantigen, enter the parenchyma of the graft, proliferate and mediate their destructive, or possibly protective, effects.

It is apparent that even though stem cell-derived tissues may not contain dendritic cells, and may not express HLA class II molecules, HLA class I-mismatched cells may be recognized both by direct pathway memory $CD8^+$ T cells and memory B cells, and by indirect pathway $CD4^+$ T cells that respond to HLA peptides presented by self class II on host APCs. It seems very likely, therefore, that stem cell-derived tissues would be susceptible to immune rejection [47].

1.7.1 T Cell Activation

Adaptive or acquired immunity to alloantigen is critically dependent upon activation of $CD4^+$ T lymphocytes that are then able to produce cytokines that provide help for differentiation of $CD8^+$ T cells and B cells, and recruitment of non-specific

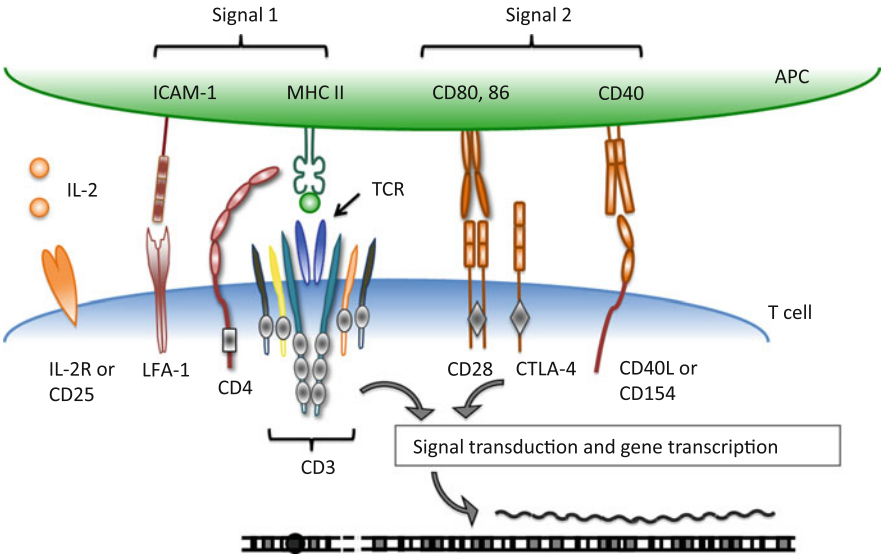

Fig. 1.10 The TCR, co-stimulation, and T cell activation. T cell activation and the induction of a graft rejection response requires two signals between T cell and APC. The initial interaction between the $\alpha\beta$ TCR and the MHC-peptide complex on the APC is stabilized by adhesion molecules (ICAM-1 with LFA-1) and the weak accessory binding of the CD4 or CD8 molecule. This results in activation of the Lck which phosphorylates ITAMs on the cytoplasmic tails of the CD3 molecule, providing Signal 1. Signal 2 is initiated by interaction between the co-stimulatory molecules CD80 and 86 on the APC with CD28 on the T cell. This induces expression of CD40 on the APC and its ligand, CD40L on the T cell. The two signals initiate subsequent signaling pathways resulting in expression of cytokine genes, particularly IL-2, whose gene product has an autocrine effect on the T cell resulting in upregulation of the IL-2 receptor and induction of T cell effector functions

inflammatory cells. T cells require two distinct signals for full activation: the first of these (signal 1) is provided by the interaction of the TCR with its specific epitope on an APC, consisting of peptide presented by self-MHC (indirect allorecognition), or an allogeneic MHC-peptide complex (direct allorecognition) (Fig. 1.10). This interaction is facilitated by the co-receptor: the CD4 or CD8 molecule that binds to a non-polymorphic part of the membrane-proximal domains of class II and class I MHC molecules respectively, enabling adhesion molecules on the two cell types to stabilize the interaction (LFA-1 on lymphocytes and ICAM-1 on APCs). Signal 1 results in partial activation of the lymphocytes, as evidenced by kinase-mediated phosphorylation of certain intracellular proteins. The second signal is provided by interaction between costimulatory molecules expressed by both T cells and activated APCs. CD28 on T cells interacts with CD80 and CD86 on activated APCs, and this induces further protein phosphorylations that complete T cell activation and initiate gene transcription leading to cytokine production and lymphocyte proliferation. Signal 1 in the absence of the second signal results in T cell anergy and apoptosis in naïve cells, but is sufficient for T cell activation in antigen-experienced lymphocytes. Expression of CTLA-4 on T cells acts as a feedback mechanism to terminate

lymphocyte proliferation, since CTLA-4 binds CD80/86 with higher affinity than CD28 and this results in the lymphocyte disengaging from the APC before intracellular signaling can occur [48].

The cascade of intracellular signaling events that follows from specific engagement of the TCR and accompanying co-stimulation is dependent upon the CD3 moiety of the TCR complex (Fig. 1.10). The CD3 molecule comprises a γ, δ, and two ε polypeptide chains, which together with the $\alpha\beta$ TCR form a complex with two ζ chains. Unlike the $\alpha\beta$ TCR, the CD3 chains have long intracellular domains which each incorporate an ITAM (immunoreceptor tyrosine-based activation motif, of which there are three on each ζ chain); on engagement of the TCR, Src-family protein tyrosine kinases (including Lck (leukocyte-specific protein tyrosine kinase) associated with the CD4 and CD8 co-receptors) are activated and phosphorylate ITAM tyrosines, recruiting ZAP-70 which, in turn, mediates further phosphorylation activity, including phosphorylation of the transmembrane adaptor protein LAT (linker for activated T cells) and the cytosolic adaptor protein SLP-76 (Src homology 2 (SH2) domain-containing leukocyte phosphoprotein of 76 kDa) [49, 50]. These adaptor proteins activate a proximal signaling complex of molecules including PLCγ1 (phospholipase C γ1) that induces the "second messenger" IP3 (inositol triphosphate) and DAG (diacylglycerol)-mediated signaling pathways necessary for T cell effector functions, expression of integrins and their receptors, and rearrangement of cytoskeletal components. DAG regulates NF-κB activation by phosphorylating the inhibitor of NF-κB, or IκB, thus permitting NF-κB to translocate to the nucleus where it has a critical function in gene transcription. The function of the IP3 second messenger pathway is broadly to regulate mobilization of intracellular calcium ions (Ca^{2+}) from the endoplasmic reticulum (ER) into the cytosol where it is the common mechanism of intracellular signaling for numerous cellular effector pathways. Intracellular free Ca^{2+} ions bind and activate a range of different calcium-binding regulatory proteins such as calmodulin which, in turn, regulates a number of effector cell functions via calmodulin-dependent protein kinases, including those regulated by DAG and thereby influencing NF-κB nuclear translocation. Ca^{2+} ions also activate calmodulin-dependent phosphatases such as calcineurin, which dephosphorylates NFAT (nuclear factor of activated T cells) and enables its nuclear translocation, where it functions as a transcription factor and mediates T cell activation via induction of the interleukin (IL)-2 gene. Another important outcome of these signaling pathways is rearrangement of the actin cytoskeleton, necessary for interaction between T cells and APCs at the point of contact or immunological synapse. This synapse consists of a peripheral ring of adhesion molecules and talin as well as a central cluster of TCRs, co-receptors, co-stimulatory molecules, and signaling elements.

Activated T cells produce the cytokine, IL-2 which is a potent "helper" factor driving and maintaining T cell proliferation and clonal expansion. It induces upregulation of the high affinity IL-2 receptor (CD25) on the same and nearby cells, enabling further IL-2 binding and differentiation into lymphocyte subpopulations secreting additional cytokines.

1.8 Mechanisms of Rejection

Although it is likely that innate immunity is an intrinsic prerequisite to the adaptive immune response, it was clearly established decades ago that the adaptive immune response, and particularly the T cell component, is essential for allograft rejection. Indeed, this dogma forms the basis of numerous transplant immunology research projects determining the contribution of individual cell types or molecules through the use of RAG knockout mice (that have no T and B cells) reconstituted with the immune cells to restore rejection.

It is now widely accepted that activation of $CD4^+$ T cells is critical to graft rejection since they are able to secrete a variety of cytokines which co-ordinate a range of potential effector mechanisms culminating in graft destruction (Fig. 1.11). IL-2 and interferon-γ are produced by the T helper 1 subset of $CD4^+$ T cells and mediate the induction of delayed-type hypersensitivity (DTH), while IL-2 is necessary for generation of cytotoxic T cells. The T helper 2 subset produces IL-4 and IL-5 which assist in driving the maturation of B lymphocytes to become plasma cells responsible for the production of alloantibody. Activated $CD4^+$ T cells under certain conditions are able to secrete additional cytokines that mediate alternative functions not necessarily associated with acute allograft rejection. For example, they may secrete the cytokine IL-10 that is characteristic of $CD25^+$ $FoxP3^+$ regulatory $CD4^+$ T cells which are critical to the normal status quo of the immune system in maintaining tolerance to self proteins, thereby avoiding the development of autoimmune disease [51]. Similarly, $CD4^+$ T cells may secrete IL-17 that has an antagonistic effect on regulatory T cells and, instead, promotes the development of autoimmunity [52]. Each of these subsets has been implicated in transplantation: regulatory T cells are associated with transplantation tolerance [53] while the T helper 17 subset of $CD4^+$ T cells (which produce IL-17) is associated with chronic allograft rejection [54].

Each of the effector mechanisms is capable of rejecting an allograft autonomously but it is common for several effector mechanisms to be recruited, either simultaneously or in sequence. Unmodified allograft rejection in rodent transplant models is characterized by a progressive heterogeneous mononuclear cell infiltrate comprising T cells, B cells, macrophages, and NK cells, and this is usually accompanied by circulating alloantibody. Typically MHC-disparate grafts undergo complete rejection within 7–14 days. The relative contribution of the different potential effector mechanisms depends on a number of variables such as the MHC disparity between donor and recipient, the type of transplant and the state of sensitization of the recipient.

1.8.1 Delayed-Type Hypersensitivity

One of the effects of the innate response is initiation of inflammation involving recruitment of non-antigen specific leukocytes, including macrophages, neutrophils, and NK cells, and secretion of cytokines and chemokines that mediate endothelial

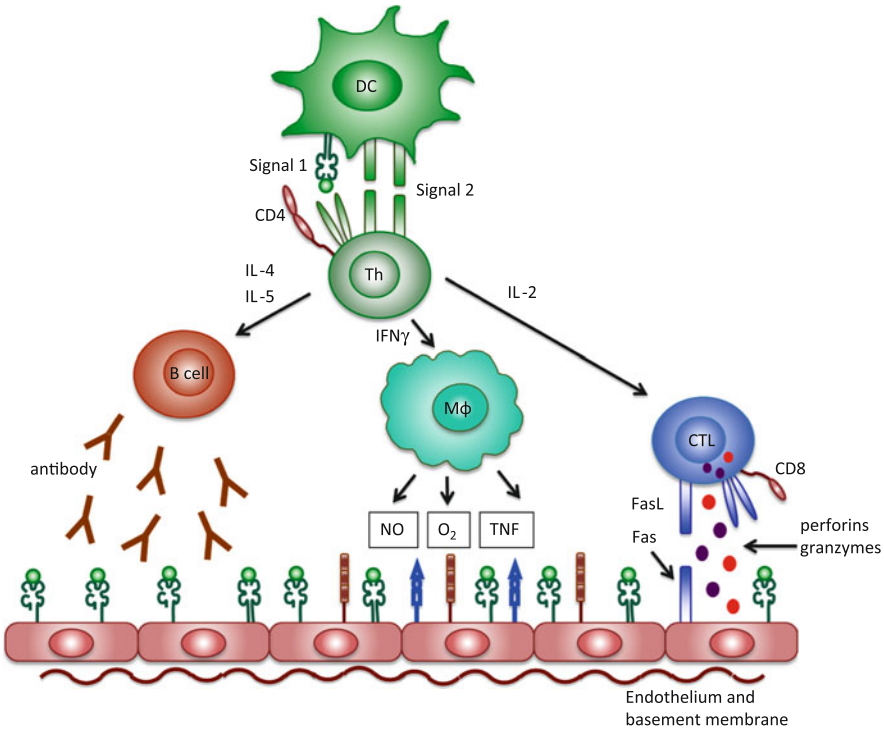

Fig. 1.11 Mechanisms of graft rejection. The $CD4^+$ T helper cell is a key player in the adaptive immune response to a transplant. $CD4^+$ T cells engage with activated APCs and are themselves activated, resulting in cytokine production that is necessary for mediating a range of effector functions. IL-5 and IL-6 are involved in maturation of B lymphocytes to plasma cells which produce graft-specific alloantibodies; IL-2 is necessary for generation of donor-specific cytotoxic T cells that lyse target cells via release of perforins and granzymes inducing apoptosis; IL-2 and IFN-γ are required for initiation of a non-specific inflammatory response or DTH reaction whereby macrophages, NK cells, and granulocytes release mediators that enhance the rejection response and cause endothelial cell activation and apoptosis

cell activation, vascular permeability, and leukocyte migration. This self-limiting response superficially resembles DTH with the exception that the DTH response is, as the name implies, not immediate like the innate inflammatory response, and depends on specific recognition of antigen by the $CD4^+$ T cell. The DTH response involves recruitment of non-specific inflammatory leukocytes, including macrophages, neutrophils, and NK cells, but also lymphocytes; as for the innate response, these cell types secrete cytokines and chemokines that mediate endothelial cell activation, vascular permeability, and leukocyte migration. The DTH skin test may be used to determine whether the individual has had prior exposure to the immunogenic agent: an intradermal injection of tuberculin purified protein derivative that causes localized erythema and induration peaking at 48–72 h is a DTH response that indicates immunity to *Mycobacterium tuberculosis*. Following organ transplantation, it is common to observe areas of inflammation within the graft that

are associated with an early perivascular infiltrate that comprises largely $CD4^+$ T cells. Activated $CD4^+$ T cells secrete cytokines (including interferon-γ and IL-2) that recruit and activate macrophages and other non-specific effector cells; tissue damage occurs through non-specific mechanisms associated with proinflammatory cytokine release (IL-1, IL-6, TNF-α) and endothelial cell activation. In clinical transplantation, such transplant DTH responses either resolve or progress to acute or chronic allograft rejection, depending on the efficacy of immunosuppression.

1.8.2 Cytotoxic T Cells

$CD8^+$ T lymphocytes have cell membrane TCRs that, as for $CD4^+$ T cells, interact with peptide-MHC complexes on the surface of APCs. $CD8^+$ T cell interactions are restricted to class I MHC-peptide complexes and, as for $CD4^+$ T cells, the interaction is stabilized by engagement of the CD8 co-receptor with a non-polymorphic region of the $\alpha 3$ domain of the class I heavy chain. $CD8^+$ T cells recognize intact allogeneic class I MHC molecules, initially on donor APCs which may deliver a co-stimulatory signal to induce maturation of $CD8^+$ T cells into cytotoxic cells with functional enzyme-containing granules (granzymes and perforins) that mediate lysis of target cells expressing the cognate ligand. As discussed above, full $CD8^+$ T cell activation requires help in the form of IL-2 secreted by $CD4^+$ T cells that recognize the same donor antigens but subsequently, antigen-experienced cytotoxic T cells are able to recognize and respond directly to class I MHC molecules that are widely expressed on the cells of donor tissues, without an additional requirement for co-stimulation or for $CD4^+$ T cell help. Unlike tissue damage mediated by a DTH response, target cell killing by cytotoxic T cells is highly specific since it is regulated by formation of the immunological synapse, or supramolecular adhesion complex (SMAC) at the point of contact between the cytotoxic cell and its target [55]. The SMAC formed between $CD4^+$ T cells and APCs is known to be prolonged, and involves signaling proteins that initiate transcription of cytokine genes. In contrast, SMAC formation in $CD8^+$ T cells occurs rapidly and is transient, comprising a peripheral ring of adhesion molecules which, through remodeling of the actin cytoskeleton form a tight seal between the two cells. In the center of the SMAC a space is formed between the two cell membranes and this is associated with polarization of the secretory components of the cytotoxic cell, including the microtubule organizing centre (MTOC), toward the SMAC. The microtubules assume a linear arrangement along which the lytic granules pass to the SMAC for release into the intracellular space. There, perforins polymerise to form pores in the cell membranes, and granzymes are then able to move from the cytotoxic T cell to the target cell cytosol. Granzymes are serine proteases that activate caspase-3 and -7, thereby triggering apoptosis or programmed cell death in the target cell. As well as granzymes, Fas ligand is also released in the target cell from lytic granules, where it complexes with the transmembrane Fas receptor at the cell surface. This complex recruits Fas-

associated death domain (FADD) that activates cytosolic caspase-8, resulting in apoptosis. Formation of the SMAC and subsequent disengagement of the cytotoxic T cell is completed within around 20 min, so that the cytotoxic cell is able to move on and engage with and kill another cell.

1.8.3 Alloantibody

It is rare, in clinical practice, for an organ transplant to fail within minutes or hours of grafting as a result of pre-existing, circulating antibodies against ABO or HLA tissue antigens because their presence is screened for in the pre-transplant cross-match test. A positive cross-match is a contraindication to transplantation, although there are now effective clinical pretreatment protocols for reducing existing antibodies to acceptable levels if there is no alternative to the transplant. However, it is now increasingly recognized that antibodies against donor HLA, and also against autoantigens, arise after transplantation and contribute significantly to acute allograft rejection [56]. A diagnosis of antibody-mediated rejection is usually made on the basis of detectable donor-specific HLA antibody in the serum together with a transplant biopsy that demonstrates (by immunohistology) deposition of the complement component, C4d, in capillaries, and accumulation of monocytes and neutrophils in graft parenchymal tissues [57, 58]. This usually accompanies some degree of mononuclear cellular infiltrate comprising $CD4^+$ and $CD8^+$ T cells and B lymphocytes, characteristic of acute cellular rejection; it is unlikely that cellular and humoral rejection are entirely functionally independent. Humoral rejection is relatively resistant to immunosuppressive treatment and persistence of circulating alloantibody is increasingly recognized to be a significant risk factor for developing chronic allograft rejection [59].

Alloantibodies mediate their damaging effects through complement-dependent and independent pathways. Fixation of complement by certain classes of immunoglobulins results in production of the chemotactic complement fragments C3a and C5a that mediate an influx of neutrophils and monocytes, as well as endothelial cell activation. The terminal components of complement, C5b-9, form the membrane attack complex which causes lysis of the target cell to which antibodies are bound. In addition, fixation of the component C6 is associated with activation of the coagulation cascade and endothelial cell injury, together with secretion of proinflammatory cytokines [60, 61]. Non-complement fixing alloantibodies may also mediate their harmful effects by activating endothelial cells to increase their expression of adhesion molecules. This may be followed by attachment of NK cells and macrophages via Fcγ receptors culminating in target cell lysis by the process termed antibody-dependent cell-mediated cytotoxicity, or ADCC [62].

Alloantibodies are generated when B lymphocyte immunoglobulin receptors with specificity for epitopes on intact donor HLA molecules engage with their target and, at the same time, receive $CD4^+$ T cell help via either a cognate or a non-cognate interaction [63, 64].

- Cognate interaction occurs when alloantigen is presented indirectly, such that donor HLA is processed and presented as peptide by recipient APCs to recipient $CD4^+$ T cells; these interact with a B cell that has internalized a soluble form of the alloantigen and is presenting the same allopeptide to the $CD4^+$ T cell.
- Non-cognate help, in contrast, is provided by $CD4^+$ T cells directly recognizing donor HLA (e.g., on donor APCs) in close proximity to B cells that are also directly engaging with donor HLA, probably on the same donor cell.

$CD4^+$ T cell help induces B cell expression of the costimulatory molecules, B7 (or CD80/86) and CD40 which serve to stabilize the interaction and promote B cell activation. While $CD4^+$ T cells can, in principle, respond to allopeptide presentation by the B cells alone, and provide help for antibody secretion, early work suggested that the B cell antibody response is massively enhanced by the presence of dendritic cells for priming CD4 help [65]. The likely explanation is that, on encountering alloantigen, dendritic cells respond rapidly by upregulating surface expression of MHC and multiple co-stimulatory molecules which not only enable formation of clusters with $CD4^+$ T cells and B cells via CD80/86/CD28 and CD40/CD154 interactions, but also facilitate an activatory environment through locally high concentrations of the cytokines IL-2, IL-4, and IL-5 secreted by the $CD4^+$ T cells.

These cell interactions occur in the secondary lymphoid tissue where a number of B cells develop into short-lived plasmablasts, secreting low affinity alloantibody, while the remainder undergo proliferation and antibody gene rearrangement in germinal centers of the lymphoid follicles [66, 67]. This process, termed affinity maturation, generates production of higher affinity antibodies by a range of clones of B cells that undergo immunoglobulin isotype switching to produce IgG class antibodies. The B cells mature to become terminally differentiated, short-lived antibody-secreting plasma cells, long-lived plasma cells that migrate via a CXCL12 chemokine gradient and persist in the bone marrow, or memory B cells, ready to respond rapidly by differentiating into plasma cells when the specific alloantigen is re-encountered subsequently. Importantly, recent evidence suggests that plasma cells are very rare, but may continue to secrete antibody for a long period of time [68]. Their persistence and turnover may depend upon the availability of specialized niches in the bone marrow and lymphoid tissue, or in inflammatory tissues [69]. In certain circumstances, B memory cells do not require a persistent specific antigenic stimulus, nor CD4 help for differentiation into plasma cells [70–73]. These findings not only partially account for the failure of conventional immunosuppressive agents to effectively control alloantibody-mediated rejection but also highlight the potential for new therapeutic strategies. Rituximab, for example, is a therapeutic antibody against the CD20 molecule developed for treatment of B cell lymphoma, and has recently been used in antibody-mediated graft rejection and in desensitization protocols in an attempt to deplete circulating antibodies. However, while CD20 is expressed by B cells, which may be effectively depleted by this agent, terminally differentiated plasma cells (that produce antibody) no longer express CD20 and are refractory to rituximab treatment [74, 75].

Continued presence of circulating alloantibodies is strongly associated with chronic allograft rejection as typified by remodeling of interstitial blood vessels and eventually, complete occlusion of the vessel lumen. Repeated cycles of antibody-mediated vessel damage and repair are thought to result in the development of multilaminate basement membranes [76]. However, under certain conditions, the persistence of low levels of circulating antibodies targeting endothelial cells can have a beneficial effect on allograft outcome. This incompletely understood phenomenon is termed accommodation and is characterized by increased expression of genes encoding complement regulatory proteins such as decay accelerating factor (DAF) and CD59, and anti-apoptotic proteins, including A20, Bcl-2, and Bcl-xL, which offer some degree of protection from antibody-mediated endothelial cell activation [77]. It is possible that signaling pathways induced by anti-HLA antibodies mediate either cellular proliferation and cytokine synthesis, or accommodation depending on the titre of anti-HLA antibodies [78].

1.9 Privileged Sites

The term immune privilege refers to the phenomenon where tissue allografts transplanted to certain anatomical sites appear exempt from the rules that normally govern graft rejection [79, 80]. Privileged sites include the cornea, the anterior chamber of the eye, the brain and the testis, and tissue allografts placed in such a site may be rejected either slowly or not at all, whereas if they are placed elsewhere in a recipient they are rejected rapidly. Moreover, tissue grafts prepared from immunologically privileged sites may enjoy prolonged graft survival when transplanted to a site where most tissue allografts are rapidly rejected. Immune privilege is thought to have evolved as a powerful naturally occurring mechanism for protecting vital tissues that are unable to regenerate from the potentially destructive effects of the immune system. The development of sperm, for example, does not begin until puberty, after immunological self-tolerance has become established, and must be protected from immune attack. The immune tolerance displayed by a pregnant mother to a fetus, which expresses paternally inherited antigens, may be regarded as another important example of immune privilege.

The mechanisms responsible for immune privilege are complex and diverse. Physiological and physical barriers, such as reduced lymphatic drainage, and the blood–brain barrier and the blood-testis barrier that depend partly on cellular tight junctions, may contribute to the protection afforded by a privileged site. However, it has become clear in recent years that immune privilege is also an active process involving a variety of different mechanisms. These include the production of anti-inflammatory cytokines, notably transforming growth factor-β (TGF-β), by APCs within the microenvironment of a privileged site, such as the anterior chamber of the eye [81]. Indoleamine 2, 3-dioxygenase (IDO) is an enzyme that is abundant at the feto-maternal interface and plays an important role in the protection of the fetus from immune attack [82]. Certain cells within immunologically privileged

tissues like the testis are able to deliver intercellular signals (e.g., via Fas–Fas ligand interaction) that induce apoptosis of cytotoxic effector cells [83, 84]. Most attention in recent years has, however, focused on the role of regulatory T cells in immune privilege, particularly "natural" regulatory T cells which are identified phenotypically as $CD4^+CD25^+$ T cells and which produce the anti-inflammatory cytokine, IL-10. Naturally occurring regulatory T cells are produced in the thymus and play an important role in maintaining tolerance to self-antigens but are also capable of regulating the graft rejection response [85]. Greatly increased numbers of regulatory T cells are found in the blood and lymph nodes draining the uterus of pregnant mice where they protect the semi-allogeneic fetuses from rejection [86].

Analogous to immune privilege, experimental approaches for avoiding activation of the recipient's immune system include encapsulation of transplanted cells within a biocompatible polymer material. This methodology has had limited success for transplantation of pancreatic islets, mesenchymal stem cells, and ES cell-derived neurons [87, 88].

1.10 Concluding Comments

The immunological basis of allograft rejection is well established and studies extending over several decades have resulted in a detailed understanding of the complex molecular and cellular mechanisms responsible for graft rejection. Advances in basic immunology and insights provided from experimental and clinical transplantation studies have, as highlighted in this chapter, enabled a comprehensive picture to be drawn, illustrating the sequence of events that follow transplantation from initial allorecognition through to destruction of a tissue graft by the diverse cellular and antibody-mediated effector mechanisms responsible for rejection. The immunological barriers to regenerative medicine have not yet been defined in any depth, but they are likely to bear many similarities with those encountered following conventional tissue and organ transplantation. Hence, the extensive experience and lessons learned from organ and tissue transplantation can now be applied to the newly emerging field of regenerative medicine.

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Chapter 2
The Immunogenicity of ES Cells and Their Differentiated Progeny

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Abstract Embryonic stem (ES) cells are an attractive source for tissue regeneration and repair therapies. This is because in contrast to adult stem cells, ES cells possess unlimited self-renewal and pluripotent capacity. However, for the therapeutic application of ES cells to succeed, the transplanted ES cells must engraft successfully and survive long enough to exert a therapeutic effect. An important obstacle facing the in vivo engraftment and function of ES cells is the immunogenic barrier. In this chapter, we will begin by briefly discussing the safety concerns regarding the transplantation of ES cells and the factors that influence the behavior or misbehavior of transplanted ES cells. We will then discuss the in vitro immunogenic properties of ES cells, including the expression of major histocompatibility (MHC) antigens and minor histocompatibility (mH) antigens and how these properties evolve as undifferentiated cells mature towards more differentiated derivatives. We will also highlight the various (and in some instances conflicting) conclusions regarding the immunogenic properties of ES cells which have been drawn from prior in vitro studies and will conclude with a more extensive discussion of the immunogenic properties of ES cells when transplanted across allogeneic as well as xenogeneic immune barriers.

2.1 Introduction

Embryonic stem (ES) cells are a promising option to regenerate tissues and organs. The ability to differentiate into different cell types has stimulated research in generating neurons [1–3], cardiomyocytes [4], hepatocytes [5], hematopoietic

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progenitor cells [6], pancreatic beta cells [7], and other cell types for potential clinical applications. This area of research is generating unprecedented interest in the scientific community because of the expectation of a new horizon in clinical medicine, but thus far it has also been plagued by ethical controversies, potentially unrealistic timelines, and practical hurdles to therapy. With respect to the latter, one of the most vexing and underappreciated problems is immune rejection of ES cells after transplantation into the recipient [8]. This occurs because ES cell-derived therapeutic cells are not "self derived" and can therefore result in an aggressive immune response from the recipient. Another potential problem with ES cell therapy is the potential of undifferentiated ES cells to form teratomas after transplantation. In this chapter, we will first review studies that have attempted to define the potential for ES cell-derived teratoma formation. We will then discuss the data characterizing the in vitro immunogenic properties of ES cells and the evidence demonstrating in vivo immune rejection of ES cells when transplanted across allogeneic and xenogeneic barriers.

2.2 Teratoma Formation

Teratomas are benign germ cell tumors, which in humans occur most often in the gonads, but may occasionally be found in extragonadal sites such as the anterior mediastinum or retroperitoneum [9]. Teratomas differ from most tumors because they are a mixture of many tissue types, whereas most tumors represent a limited diversity of neoplastic cell types. Histological evaluation of a teratoma by definition will demonstrate tissues from all three embryonic germ layers, that are haphazardly arranged throughout the tumor in a way that partly resembles a disorganized embryo [10]. There presently exists no method capable of generating a 100 % pure population of differentiated cells from a pluripotent donor source. Therefore, it is exceedingly difficult to confirm that a preparation of therapeutic cells is not contaminated by residual pluripotent ES cells that have escaped the differentiation process and, consequently, teratoma development is of significant clinical concern [11]. The potential for teratoma formation is influenced by multiple factors, including the immune system [12], transplanted cell number [13], and graft site [14]. The influence of cell number on the potential for teratoma formation is clinically relevant because it establishes a threshold by which to gauge the number of contaminating undifferentiated ES cells that may reliably produce teratoma formation upon transplantation. A previous report investigating the relationship between human ES (hES) cell number and teratoma formation demonstrated that consistent teratoma formation in immunodeficient mice depends both on cell number and transplantation site [13]. Teratoma formation upon transplantation in the myocardium and the skeletal muscle requires $\sim 1 \times 10^5$ and $\sim 1 \times 10^4$ hES cells, respectively. This suggests a critical threshold for the number of undifferentiated hES cells to produce teratoma formation. Additionally, the in vivo graft site can influence the propensity of

transplanted hES cells to remain in the undifferentiated state. This is exemplified by the observation that when hES cells are intrahepatically versus subcutaneously transplanted into immunodeficient mice, the number of cells that remain undifferentiated and the kinetics of teratoma formation are both enhanced [14]. It is thought that the highly vascular and growth-factor rich environment of the liver may explain why hES cells are less prone to differentiation when transplanted intrahepatically versus subcutaneously [14]. Although these studies involved immunodeficient mice, they illustrate the influence that cell number and transplant location exert on ES cell survival, differentiation, and behavior. As will be discussed in more detail, the literature regarding the immunogenicity of ES cells is rather controversial because there exist numerous reports that have drawn directly conflicting conclusions. When the immunogenicity of ES cells is evaluated based on cell survival in immunocompetent animals, differences in experimental design similar to those mentioned above may help explain the conflicting conclusions.

2.3 Cell Surface Expression of Immunogenic Molecules

Numerous groups have attempted to characterize the immunogenic properties of hES cells by assaying their surface expression of potentially "immunogenic" molecules. The first study to do so focused on the expression of major histocompatibility complex (MHC) antigens. MHC antigens are a critical group of antigens that are classically associated with transplant rejection. MHC antigens are divided into class I, which are expressed by most human cells, and class II, which are generally restricted to antigen presenting cells, macrophages, and B-cells [15]. In the undifferentiated state, hES cells express low levels of MHC class I and minimal levels of MHC class II [16]. However, exposing hES cells to IFN-γ induces the expression of MHC class I. Similarly, allowing the cells to undergo spontaneous differentiation into embryoid bodies (EB), which are three-dimensional structures composed of an amalgam of hES-derived cell types, stimulates increased MHC class I expression [16] (Fig. 2.1). Interestingly, the expression of MHC class II and co-stimulatory molecules (e.g., CD80, CD84, CD40) by hES cells appears to be very low, and neither incubation with IFN-γ nor spontaneous differentiation into EBs induces any substantial increases in the expression of these proteins [17]. The hES cell expression of negative immunoregulatory proteins and cytokines has also been assessed. hES cells do not express the cell surface protein CD95 ligand (Fas ligand), which is a known inducer of apoptosis [18] nor do they secrete the immunosuppressive cytokine interleukin-10 [19]. Thus, in the differentiated state, hES cells possess a comparable cell surface expression pattern to the majority of human cells (e.g., fibroblasts), consisting of MHC class I but not MHC class II, costimulatory molecules, and negative immunoregulatory molecules.

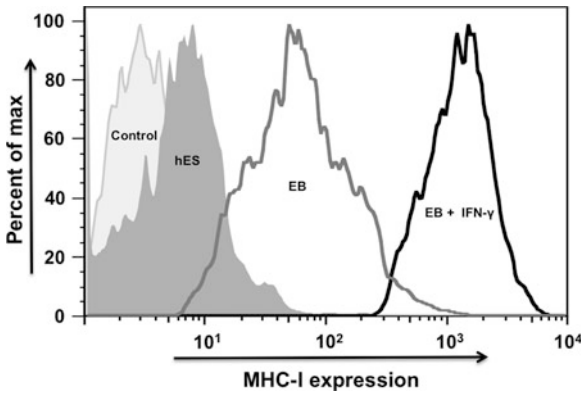

Fig. 2.1 Florescent activated cell sorting analysis of MHC class I expression by hES cells and their differentiated derivatives. The MHC class I expression increases as hES cells differentiate or are incubated with IFN-γ. Control = isotype matched control, hES = undifferentiated hES cells, EB = hES-derived embryoid bodies, EB + IFN-γ = EBs incubated with IFN-γ

2.4 In Vitro Immunogenic Properties

To evaluate the in vitro immune response towards hES cells, previous groups have performed mixed leukocyte reactions (MLR), with hES cells serving as the immune "stimulator" cell population. The results from these in vitro assays have produced conflicting results regarding the severity of the immune response elicited by hES cells. One study demonstrated that hES cells do not stimulate proliferation of allogeneic human peripheral blood mononuclear cells (hPBMC), nor do hES cells stimulate proliferation of allogeneic human peripheral blood lymphocytes (hPBL) [17]. Similarly, when the hES cells were differentiated into EBs or incubated with IFN-γ, only minimal T cell proliferation was observed. These results seem to indicate that in the undifferentiated state, when they have only marginal MHC class I expression, hES cells induce limited immune stimulation. Surprisingly, when the hES cells differentiate and increase their expression of MHC class I, they still do not provoke immune activation. These results led to the suggestion that hES cells might possess unique immune privileged characteristics [17]. In addition to evidence indicating that hES cells do not themselves activate responder leukocytes, there is evidence suggesting that hES cells actively inhibit the activation of responder leukocytes. Specifically, it has been shown that the inclusion of hES cells in a MLR involving responder hPBL and allogeneic stimulator dendritic cells results in decreased hPBL proliferation compared to allogeneic dendritic cells and hPBMCs alone [17]. This indicates that hES cells actively inhibit the allogeneic T cell response towards third party antigens.

In contrast, other studies using MLRs to characterize the immunogenicity of hES cells have reached opposite conclusions. A MLR using human $CD4^+$ T cells and dendritic cells from the same donor, mixed with allogeneic hES cells,

demonstrated not only that hES cells lack an inhibitory effect on T cell proliferation, but that hES cells induce T cell proliferation [20]. The level of T cell proliferation stimulated by the hES cells was comparable to that induced by allogeneic human fibroblasts, but it was four-fold less than that induced by allogeneic dendritic cells. This may be because hES cells and fibroblasts express MHC class I, but both lack expression of MHC class II and costimulatory molecules, whereas mature dendritic cells display MHC class I, MHC class II, and costimulatory molecules such as CD80, CD86, and CD40 which confer upon them the potent capacity for T cell activation.

These two studies attempted to define the in vitro immunogenic properties of hES cells using MLRs and arrived at contradictory conclusions. The conflicting results likely reflect heterogeneity in experimental design between the two studies. Using a different in vitro approach, a third group concluded that hES cells possess a level of immunogenicity that is intermediate to that described by the two studies discussed above. They found that primed cytotoxic T lymphocytes (CTL), have the capacity to recognize and lyse hES cells if the hES cells are rendered sufficiently immunogenic [21]. In this study, CTLs were primed to recognize HLA-A2 antigens because hES cell lines H9 and H13 express HLA-A2. The CTLs were primed by co-culture with irradiated hPBMCs expressing HLA-A2 and loaded with influenza type A peptide (IV/A). When the primed CTLs were mixed with hES cells loaded with IV/A, the CTLs did not lyse the hES cells. However, when the hES cells were infected with influenza virus and MHC class I expression was induced by incubation with IFN-γ, efficient CTL mediated lysis was observed. This indicates that with the proper peptide-loading method and sufficient expression of MHC class I, CTLs can recognize and lyse hES cells.

2.5 Allogeneic Transplantation of ES Cells

Much of our understanding regarding the immunogenic properties of ES cells has come from the study of mouse ES (mES) cells transplanted into a murine host, because this represents an allogeneic in vivo transplantation scenario. Whether the conclusions drawn from the mouse model system can reliably be extrapolated to the human scenario remains to be determined. One of the first reports indicating that ES cells may be immunogenic involved intramyocardial transplantation of mES cells following myocardial infarction [22]. The allogeneic graft site was infiltrated by a significant cellular infiltrate composed of T cells and dendritic cells, and analysis of the host sera demonstrated the presence of alloantibodies. This cellular and humoral immune response was progressive, increasing in intensity from 1 to 4 weeks following transplantation and correlated with the increased expression of MHC class I antigens by mES cells [22].

A similar study involving transplantation of mES cells into ischemic myocardium demonstrated that the allogeneic immune response is of sufficient intensity to prevent the long-term engraftment of mES cells across histocompatibility

barriers [23]. Allogeneic mES cell grafts incited a mild $CD4^+$ T cell dominated inflammatory infiltrate at 1 week post transplantation, which progressed towards a severe inflammatory infiltrate composed of both $CD4^+$ and $CD8^+$ T cells at 4 and 8 weeks after transplantation. In contrast, syngeneic mES cell grafts produced a limited inflammatory infiltrate that was comparable to the sham procedure group at all time points. At 8 weeks after transplantation, mES cells were still detected in syngeneic recipients. By comparison, no evidence of allogeneic mES cell engraftment was observed. The above results were confirmed by a different group that similarly investigated mES cell survival after intramyocardial transplantation into allogeneic and syngeneic recipients [24]. At 3 weeks post transplantation, the inflammatory infiltrate was significantly greater in allogeneic compared to syngeneic grafts. At later time points, the allogeneic grafts were completely rejected, whereas the syngeneic grafts survived indefinitely [24]. In regard to the immunogenicity of mES cells, when mES cells are transplanted across histocompatibility barriers, engraftment will be significantly limited by the host alloimmune response.

If ES cells are recognized as antigenic by the host adaptive immune system, the host will generate immune memory cells with specificity towards these antigens. Upon future exposure to the antigens, these memory cells will orchestrate a more rapid and robust immune response. This was previously demonstrated for mES cells transplanted into MHC-mismatched hosts [12]. When mES cells were intramuscularly transplanted (gastrocnemius muscle) into syngeneic recipients, intramuscular teratoma formation was observed in all recipients by day 28. In contrast, no evidence of mES cell survival was observed in allogeneic recipients at day 28, presumably because of alloantigen specific rejection of the transplanted mES cells [12]. To test if immunologic memory was induced towards mES cells, the kinetics of the secondary immune response were compared to this primary immune response. Upon repeated exposure, mES cells were rejected by day 7, demonstrating an accelerated immune response relative to the 21–28 days required for rejection during the primary exposure [12]. This indicates that immune memory cells are generated as a result of the adaptive immune response against allogeneic mES cells.

The immunogenicity of ES cells may also depend on the differentiation state of the graft. When ES cells differentiate or are exposed to an inflammatory environment (e.g., IFN-γ), MHC class I expression is increased [12, 16, 17]; this may result in a heightened allogeneic immune response to the ES cells. This was addressed by two experiments comparing the survival of undifferentiated and differentiated mES cells transplanted into MHC-mismatched hosts [12]. The first experiment demonstrated that undifferentiated mES cells are immunologically rejected by day 28, but if the cells are allowed to first differentiate in vivo and are then isolated and re-transplanted, they are rejected by day 14. The second experiment demonstrated that if a very large number ($\sim 1 \times 10^7$) of undifferentiated mES cells are transplanted into allogeneic recipients, a minority of the grafts (~ 20 %) overcome immune rejection and form teratomas [12]. However, if the mES cells are first allowed to differentiate in vitro prior to transplantation, none of the grafts will escape immunological rejection. This accelerated cell death and

diminished survival may reflect increased immunogenicity of the differentiated mES cells, but is also compatible with the view that when the cells differentiate, their proliferation rate decreases and they become more vulnerable to the immune response than the highly proliferative undifferentiated cells.

The previous examples have demonstrated that transplantation of mES cells across allogeneic MHC barriers can result in immune-mediated rejection. However, in addition to MHC antigens, ES cells express minor histocompatibility (mH) antigens that may contribute to their immunogenicity. The potential impact of mH antigens was investigated by transplanting mES cell-derived EBs into mH antigen mismatched, but MHC matched hosts [25]. EBs which differed only at mH loci were rejected with similar kinetics as both fully MHC-mismatched EBs and MHC-mismatched skin grafts [25]. This finding demonstrates that mES derivatives may be as vulnerable to immune rejection as other types of grafts. Furthermore, it indicates that matching donor and host MHC antigens may not be sufficient to prevent graft rejection, and thus some form of immune intervention will likely be necessary.

2.6 Xenogeneic Transplantation of ES Cells

Similar to the mES cell studies, mixed conclusions have been reached regarding the immunogenicity of hES cells. Due to ethical constraints, the in vivo immunogenic properties of hES cells have not been studied in a true allogeneic scenario (i.e., human transplantation). Instead the majority of studies have either investigated the immunogenicity of hES cells in vitro or in the xenogeneic transplantation setting using rodents as the experimental host.

One of the earlier studies suggesting that hES cells may possess some form of immune privilege involved transplantation of hES cells into the quadriceps muscle of immunocompetent mice. Using histopathological techniques, the investigators were unable to detect an appreciable inflammatory infiltrate at 24 and 48 h after injection. This finding indicates that hES cells may not induce a significant inflammatory infiltrate at the early time points assayed. However, studies which assayed *later* time points demonstrated signs of immune-mediated rejection by 3 days with escalating intensity at 5–7 days post transplantation [20]. The inflammatory cells which infiltrate the hES cell graft are predominantly T and B-cells, indicating the involvement of the adaptive immune system [26]. However, neutrophils and macrophages are also present, likewise suggesting the involvement of the innate immune system [26]. To demonstrate that the immune rejection of xenogeneic hES cells was not unique to a certain mouse strain, a different group of investigators transplanted hES cells into 4 different immunocompetent mouse strains. They found that at 1 month post transplantation, every immunocompetent animal rejected the hES cells, whereas all immunodeficient mice accepted the grafts and demonstrated teratoma formation [21]. The rejection of hES cells appears to be predominantly orchestrated by the $CD4^+$ T cell subset. When hES cells were transplanted into $CD4^{-/-}$ mice, their survival was significantly

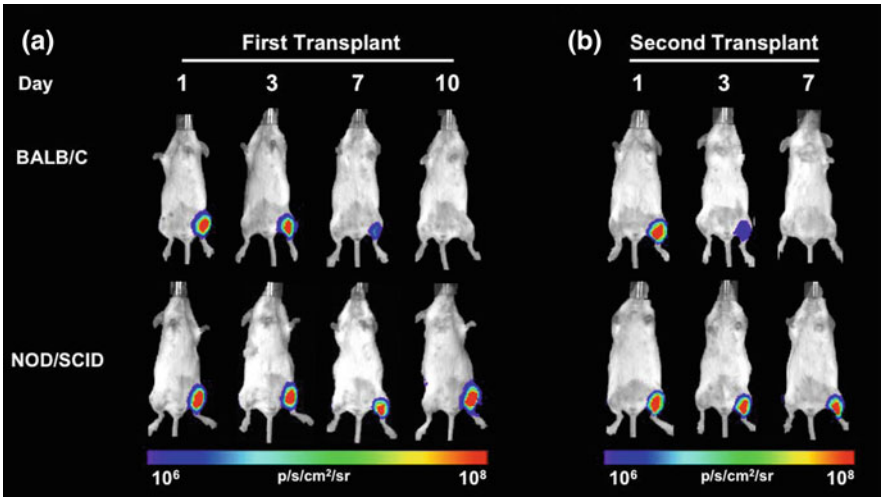

Fig. 2.2 In vivo visualization of hES cell survival. **a** Representative bioluminescent images (BLI) of hES cell transplanted naïve animals (*first transplant*) shows a rapid decrease in BLI signal in immunocompetent (BALB/c) mice, compared to immunodeficient NOD/SCID mice, reaching background levels at day 10 after transplantation. **b** In BALB/c animals that were presensitized with nontransduced hES cells (injection in *right leg*), accelerated loss of the BLI signal was seen upon second transplantation (injection in *left leg*) due to prior pre-sensitization, reaching background intensity by day 7. Color scale bar values are in photons per second per square centimeter per steradian (p/s/cm^2/sr)

prolonged relative to that observed in CD8$^{-/-}$ mice [26]. Interestingly, hES cells were eventually immunologically rejected (albeit with differing kinetics) and failed to engraft in both CD4$^{-/-}$ and CD8$^{-/-}$ mice, indicating that either T cell subset was sufficient to prevent hES cell engraftment. However, T cell deficient nude mice were unable to reject hES cells, indicating that at least one of the two T cell subsets was necessary for hES cell rejection [26].

The xenogeneic immune response towards hES cells is an adaptive immune response that consists of both a humoral and cellular arm. Murine splenocytes that have been exposed to hES cells in vivo produce significantly increased levels of both IFN-γ and IL-4 cytokines compared to splenocytes isolated from naïve mice never exposed to hES cells [26]. IFN-γ is produced by T-helper type-1 (Th1) cells which classically induce a cellular immune response. IL-4 is produced by T-helper type-2 (Th2) cells which facilitate the humoral immune response. Indeed, following hES cell transplantation, there are increased quantities of xeno-reactive antibodies in recipient sera relative to control mice. Further proof that hES cells can elicit an adaptive immune response is that, like mES cells, hES cells can stimulate the production of immune memory cells. Primary transplantation of hES cells will result in the complete immunological rejection of xenogeneic grafts by 7–10 days post transplantation. If the same animals are transplanted 14 days after primary challenge with the same number of hES cells, the secondary immune response will accelerate hES cell death and produce complete immunologic

Table 2.1 Summary of previous studies demonstrating immune rejection of transplanted ES cells

Cell type	Transplant type(s)	Transplant site	Results	Ref
mES cells: undifferentiated	• Syngeneic • Fully allogeneic	Intramyocardial	• Increasing infiltration of graft by inflammatory (T and Dendritic) cells from 1–4 weeks • Increasing humoral antibody response from 2–4 weeks • Allogeneic graft incited a Th-1 type immune response consisting of IFN-γ and IL-2	[22]
mES cells: undifferentiated	• Syngeneic • Fully allogeneic	Intramyocardial	• At 4 weeks teratoma formation was apparent in both syngeneic and allogeneic hosts • At 8 weeks teratomas persisted in syngeneic hosts, but no cells were detected in allogeneic hosts • Increasing infiltration of allogeneic grafts by inflammatory cells from 1–8 weeks	[23]
mES cells: undifferentiated	• Syngeneic • Fully allogeneic	Intramyocardial	• Teratoma formation in syngeneic hosts at 3 weeks which remained at 5 weeks • Teratoma formation in allogeneic hosts at 3 weeks but 90 % of grafts rejected at 5 weeks • Progressively increasing infiltration of allogeneic grafts by inflammatory cells from 3–5 weeks	[24]
mES cells: embryoid bodies	• Syngeneic • Fully allogeneic • mH antigen mismatch	Kidney capsule	• Syngeneic grafts became well vascularized and increased in size • Fully allogeneic grafts were infiltrated by T cells and macrophages and rejected in all recipients by day 16 • Grafts which differ solely at mH antigens were rejected at a rate similar to fully allogeneic grafts	[25]
mES cells: undifferentiated and differentiated	• Syngeneic • Fully allogeneic	Intramuscular	• Syngeneic grafts develop into teratomas by day 28 • Allogeneic grafts are fully rejected by day 28 upon primary and by day 7 upon secondary transplantation • Undifferentiated allogeneic cells are rejected by day 28, whereas differentiated grafts are rejected by day 14	[12]

(continued)

Table 2.1 (continued)

Cell type	Transplant type(s)	Transplant site	Results	Ref
hES cells: undifferentiated	• Xenogeneic	Intramyocardial	• Xenogeneic grafts were infiltrated by T cells and macrophages • Xenogeneic grafts were rejected by day 4 in immunocompetent mice	[20]
hES cells: undifferentiated	• Xenogeneic	Kidney capsule	• Xenogeneic grafts were rejected by 1 month in all 4 mouse strains tested	[21]
hES cells: undifferentiated and differentiated	• Xenogeneic	Intramuscular	• Grafts were infiltrated by T cells ($CD4^+$ and $CD8^+$), B-cells and neutrophils • Xenogeneic grafts elicited a Th-1 type (IFN-γ) and Th-2 type (IL-4) immune response • Donor grafts were undetectable by day 7 upon primary and day 3 upon secondary transplantation	[26]

rejection by day 3 after transplantation [26] (Fig. 2.2). This indicates that hES cells were recognized as antigenic upon primary exposure, leading to the generation of immune memory cells that produced an accelerated adaptive, donor-specific immune response upon secondary immune challenge.

2.7 Conclusion

The pluripotent capability of ES cells highlights their potential for future therapeutic applications in regenerative medicine to treat numerous intractable illnesses. However, this pluripotency also underlies the potential risk of teratoma formation if undifferentiated cells are transplanted. Similarly, the immunogenicity of ES cells represents one of the major barriers precluding the successful translation of ES cell based therapies. The immunogenic characteristics of ES cells are dynamic and in constant flux depending on the differentiation state and environment surrounding the ES cells. When ES cells are in the undifferentiated state their high proliferation rate and low expression of potentially immunogenic surface proteins presents an elusive target for the immune system. However, when the cells differentiate and increase their expression of immunogenic cell surface markers, they are placed at increased risk for immunologic rejection. This risk for immune rejection has been demonstrated for mES cells in the allogeneic in vitro and in vivo setting and for hES cells in the allogeneic in vitro and xenogeneic in vivo scenario (Table 2.1). A critical area of investigation for the future success of regenerative medicine will focus on strategies to combat immunological rejection or to induce immunologic tolerance towards ES cells. For the successful development of these approaches, investigators must identify the antigenic components of ES cells that contribute to their immunogenicity, as well as gain a better understanding of the in vivo behaviour of ES cells.

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Chapter 3
Interaction of Embryonic Stem Cells with the Immune System

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Abstract Embryonic stem (ES) cells interact with the immune system in unique ways. Immune interactions of ES cells and tumor formation appear to be reciprocal functions—the less immunity ES cells provoke, the greater the risk of tumor formation. Knowledge of the interaction of ES cells and their derivatives with the immune system and their relationship to tumor formation is critical to their potential therapeutic applications to regenerative medicine.

3.1 Introduction

Embryonic stem (ES) cells interact with the immune system in unique ways that may favour the spontaneous formation of tumours. Whereas the need to understand and overcome the immunological barriers to using allogeneic ES cells is essential for the treatment of degenerative diseases, overcoming such obstacles may inadvertently generate conditions in which ES cells and their progeny become tumorigenic [1].

In principle, every developing fetus once contained ES cells that could stimulate alloimmunity and the rejection that ensues or, in the absence of alloimmunity, generates tumors. That neither occurs regularly in nature suggests that cellular

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interactions within the fetus, trophoblast, and/or mother powerfully halt these processes. The mechanisms preventing rejection of the semi-allogeneic fetus and formation of tumors have been reviewed previously [2, 3]. Here, we consider the impact of ES cells and their derivatives on the innate and adaptive immune systems and the fate of these cells early after transplantation as a cellular graft. To understand the basis of these interactions, however, it is useful to consider the general properties of alloimmune responses and of ES cells and how these properties relate to the questions posed.

The immune response to allografts (transplants consisting of cells, tissues or organs from one individual of a species into a different individual of the same species) exhibits three unique properties. Alloimmune responses, as such, are universal, rapid, and extraordinarily destructive. We have discussed in detail the basis of these properties previously [4, 5].

We describe the immune reaction to allotransplantation as universal (it occurs in every unmanipulated recipient against every type of cell bearing foreign major histocompatibility complex (MHC) antigens), rapid (it occurs in days), and quite severely destructive (it completely destroys the target) [6]. In contrast, immune responses to bacteria, viruses, fungi, vaccines, and other antigens, including minor histocompatibility (mH) antigens, occur sporadically over periods of weeks or even months and do not generally destroy the targeted cells, at least not rapidly. The difference between the immune response to allografts and to all other antigenic challenges could reflect the peculiar way in which MHC antigens are presented or it could reflect unanticipated immunological memory, memory responses, like allogeneic responses, being universal, rapid, and highly effective [7, 8].

The mechanism by which components of the immune system, particularly T cells, recognize allogeneic cells might explain the universal, rapid, and highly destructive allogeneic response. T cells can recognize allogeneic cells “directly”, that is T cells recognize intact allogeneic MHC molecules expressed on allogeneic antigen presenting cells (APCs). By direct recognition, a T cell can potentially engage a large fraction of a given MHC on APCs (because peptide plays a nominal role) [9] or because the receptor can recognize many peptides [10]. Hence, direct recognition activates up to 10 % of T cells [9, 11] and responses can be used to test for MHC class II expression and to map MHC [12]. In contrast, T cells recognize other antigens, such as toxins, bacterial and viral proteins, as degraded foreign peptides associated with MHC on autologous APCs. When T cells recognize antigen “indirectly” on autologous APCs, only a small fraction of MHC complexes contain a given peptide. Hence, indirect recognition activates only a small fraction of one percent of T cells; in some cases no activation ensues.

Although the difference between direct and indirect recognition has been the subject of much comment, and may be important for the biology of allografts in some cases, this difference does not fully explain why allograft rejection is universal, rapid, and severe. Grafts consisting of allogeneic cells and tissues are fed by blood vessels of the recipient and the immunological reaction seems to be

directed mainly against these blood vessels [13, 14]; recognition of these blood vessels must involve the indirect pathway, that is, peptide of the donor presented by MHC of the recipient. Yet, these grafts are rejected universally, rapidly, and severely. Nor does eliminating allogeneic MHC from the surface of all donor cells prevent or even slow the course of allograft rejection [15].

If peptides derived from allogeneic MHC molecules presented indirectly can stimulate powerful rejection responses, then the immune system might recognize peptide of allogeneic MHC differently from peptides derived from other proteins. Before association of peptide with MHC had been proven, Jerne [16] speculated that the immune system is predisposed to respond aggressively to allogeneic MHC. Heightened immunogenicity of MHC-derived peptides must reflect, at least in part, the efficiency with which peptides derived from allogeneic MHC molecules are loaded on endogenous MHC determinants [17]. We found that T cells may be selected by peptides such as those from immunoglobulin molecules, encoded by the immunoglobulin supergene family [18].

As still another explanation for the universal, rapid, and severe response to allotransplantation, one might postulate that the allogeneic response is actually a manifestation of immunological memory. Consistent with this possibility, many of the T cells that respond to allogeneic cells in human adults are memory T cells [19]. Furthermore, allogeneic grafts in the newborn sometimes generate immunity and sometimes do not [20]. Also, consistent with this concept, newborn mice do not reject tumor grafts acutely but can still be primed to generate second set of responses [21]. On the other hand, Billingham et al. [22] found that young rabbits reject skin as vigorously as mature rabbits.

The universal, rapid, and severe responses to the allogeneic cells proved critical in the discovery and mapping of the MHC. Hence, it is particularly striking that pluripotent stem cells of the mother and the fetus, and the fetus itself, appear to violate these properties [2]. Whether and how ES cells also violate the general nature of alloimmune responses is discussed below.

3.2 Characteristics of ES Cells

ES cells have three generally recognized properties: (1) they derive from the inner cell mass of the embryo; (2) they proliferate indefinitely in culture; and (3) they have the capacity to differentiate into any type of cell in the body, including germ cells [23–25]. Of these properties, the capacity to differentiate has proven most controversial.

Because of the capacity to differentiate into any somatic cell, ES cells are considered pluripotent. ES cells are not considered by most to be totipotent, because they are not thought to contribute to the extra-embryonic trophoblast [26]. Beddington and Robertson [26] injected murine ES cells into blastocysts and found that they contributed to all tissues of the fetus but contributed very little to trophoblasts and tissues derived from primitive endoderm. However, this matter is

controversial [27, 28]. Thomson et al. [27] found that ES cells from primates secrete chorionic gonadotrophin into the medium after in vitro differentiation, suggesting that at least some of the cells differentiate into trophoblast cells. Using ES cells engineered to express Oct-3/4 under the control of a tetracycline transactivator, Niwa et al. [28] found that repression of Oct-3/4 induces murine ES cells to differentiate into trophoblast cells in vitro.

Primordial germ cells also generate pluripotent stem cells and some refer also to these cells as "embryonic" stem cells [29]. However, pluripotent stem cells isolated from primordial germ cells require different culture conditions to remain undifferentiated (i.e., fibroblast growth factor 4) compared to pluripotent stem cells derived from the embryo and, will not, therefore, be considered "embryonic" stem cells here [29–32].

3.2.1 Isolation of Embryonal Carcinoma Cells

Early concepts of pluripotency appear to have emerged from the study of human ovarian teratomas, which are tumors that contain well differentiated somatic cell types of all three germ layers interspersed with undifferentiated cells called embryonal carcinoma cells [33]. In 1907, Askanazy [34] studied the histology of human ovarian teratomas and hypothesized that the well differentiated somatic cell types might arise from a single pluripotent stem cell. Jackson and Brues [35] studied murine ovarian teratocarcinomas and noted that undifferentiated areas of the tumors contained more cells undergoing mitosis compared to the more differentiated areas, supporting the idea that an undifferentiated pluripotent stem cell might give rise to the differentiated cell types in the tumors.

At the time, the identity of the pluripotent stem cells hypothesized by Askanazy [34] to give rise to teratocarcinomas was unknown. In an effort to identify the cells that gave rise to teratocarcinomas, Pierce et al. [36] transplanted embryoid bodies, which are cystic aggregates containing only two or three types of cells including embryonal carcinoma cells into mice and found that teratocarcinomas developed in some of the recipients. More importantly, Pierce et al. [36] found that the frequency of teratocarcinoma formation after transplantation of embryoid bodies was directly related to the percentage of embryonal carcinoma cells, providing indirect evidence that embryonal carcinoma cells were the pluripotent stem cells giving rise to teratocarcinomas. Kleinsmith and Pierce [37] provided the first direct evidence that embryonal carcinoma cells are pluripotent and responsible for teratocarcinoma formation. Kleinsmith and Pierce [37] isolated embryonal carcinoma cells from murine teratocarcinomas and found that cloned lines of these cells could become teratocarcinomas after transfer into syngeneic mice. Finch et al. [38] were the first to establish cultures of embryonal carcinoma cells that remained pluripotent. They isolated embryonal carcinoma cells from teratocarcinomas and found that if the cells were cultured on embryonic feeder layers the embryonal carcinoma cells would remain undifferentiated for as long as 9 months.

3.2.2 Pluripotent Stem Cells of the Early Embryo

The first evidence that embryos contain pluripotent stem cells came from studies in which fertilized murine zygotes were transplanted to ectopic sites. Runner [39] found that 1 of 28 murine zygotes transplanted into the anterior chamber of the eye developed into cells representing the three germ layers but later regressed, presumably because the cells were rejected. Stevens [40] transplanted zygotes and early stage embryos (up to 8 cells) into the testicles of syngeneic mice and found that early stage embryos but not the zygotes form teratomas, suggesting that pluripotency may be restricted to certain times in development. Damjanov et al. [41] found that murine embryos harvested 6 or 7 days after fertilization formed teratocarcinomas after transplantation beneath the kidney capsule, while embryos older than 7 days did not. Solter et al. [42] tested whether the pluripotent cells in the embryo were derived from extraembryonic tissues or the portion of the embryo which gives rise to the fetus, called the embryo proper. Thus, they transplanted 8-day-old murine embryos or their extraembryonic tissues under the kidney capsule of syngeneic mice and found that the embryo, but not the extra-embryonic tissues, formed teratocarcinomas. These studies demonstrated that cells from the early embryo, but not from extraembryonic tissues, are pluripotent and that this property is lost early in life.

Pluripotent cells of the early embryo share many characteristics with embryonal carcinoma cells [43]. Pluripotent cells from the early embryo form teratocarcinomas indistinguishable from tumors formed by transplantation of embryonal carcinoma cells [42, 44–46]. On the other hand, embryonal carcinoma cells injected into blastocysts can participate in normal embryogenesis. For example, Papaioannou et al. [47] injected embryonal carcinoma cells into 3.5–4-day-old blastocysts and found that the embryonal carcinoma cells contributed to numerous tissues in chimeric mice. Consistent with the plasticity of embryonal carcinoma cells are experiments revealing that pluripotent cells from the early embryo have a similar phenotype to embryonal carcinoma cells. Bernstine et al. [48] found that both early embryos and embryonal carcinoma cells express high levels of alkaline phosphatase. Artzt et al. [49] found that the anti-embryonal carcinoma antibodies recognize the cells of 2–8-cell embryos as well as the embryonal carcinoma cells, but failed to recognize differentiated cells or zygotes.

The observation that cells of the early embryo exhibit pluripotency and a phenotype similar to embryonal carcinoma cells suggested they might be maintained in an undifferentiated state, like embryonal carcinoma cells [50]. However, the pluripotent stem cells of the embryo proved more difficult to isolate and culture than embryonal carcinoma cells. This difficulty was ascribed to one or more of three factors [50]. First, the number of pluripotent cells in the embryo is small, too small to survive initial culturing. Second, pluripotent stem cells exist only transiently and hence can be missed. Third, unlike embryonal carcinoma cells, pluripotent cells differentiate rapidly in culture. The problem was solved in part when Solter and Knowles [51] developed a method to efficiently isolate cells from

the inner cell mass by treating blastocysts with rabbit anti-mouse serum and guinea pig complement to kill trophoblast cells while not harming cells of the inner cell mass. However, the stem cells isolated by this method either did not attach to the culture dish or differentiated rapidly and hence could not be studied optimally. Sherman [52] questioned whether the failure to isolate pluripotent cells from embryos reflected suboptimal culture conditions or the absence of a growth factor. To address that question, Sherman [52] cultured two- and four-day-old embryos under various conditions, observing that the outgrowths of the inner cell mass varied widely in morphology. Cells from some embryos could be cultured for more than a year; however, the cells did not exhibit pluripotency. To determine whether failure to maintain pluripotency reflected absence of a growth factor, Atienza-Samols and Sherman [53] cultured cells isolated from the inner cell mass with medium conditioned by various cell types. Medium conditioned by embryonal carcinoma cells or blastocysts further improved outgrowths from inner cell mass but did not generate pluripotency.

3.2.3 Derivation of Human ES Cells

The derivation of ES cells from non-human primates helped to establish methods that would eventually be applied to the isolation of human ES cells. Thomson et al. [27] derived ES cells from a 6-day-old blastocyst from a rhesus monkey. They noted that monkey ES cells differed from murine ES cells in the expression of certain gangliosides. The monkey ES cells expressed SSEA-3 and SSEA-4, which are not expressed on murine ES cells, while the monkey ES cells did not express SSEA-1, which is expressed by murine ES cells. The cell culture requirements for monkey ES cells also differed from those of murine ES cells. Leukemia inhibitory factor (LIF) did not prevent differentiation of monkey ES cells in cultures lacking feeder layers while it did prevent differentiation of murine ES cells cultured without feeder layers.

The distinct methods needed for isolation and culture of ES cells from monkeys helped to explain why efforts to isolate human ES cells had failed. Bongso et al. [54] tried to isolate human ES cells from in vitro fertilized eggs but failed because the fertilized eggs were cultured without feeder layers and with LIF, as a result of which the cells differentiated after two passages. Thomson et al. [27] suggested that human ES cells might be isolated similarly to monkey ES cells, and ultimately, human ES cells were successfully established in culture from in vitro fertilized embryos using murine embryonic fibroblast feeder layers and no LIF [55].

3.2.4 ES Cells and the Treatment of Disease

ES cells and their derivatives are thought to be potentially useful for the treatment of disease. One potential therapeutic strategy would involve administration of undifferentiated ES cells into a tissue within which the cells might undergo differentiation. Hodgson et al. [56] injected undifferentiated rat ES cells into infarcted myocardium and found that the cells differentiated into cardiomyocytes and integrated into the myocardium at the site of infarction. The procedure improved left ventricular ejection fraction compared to sham-treated rats. Yamada et al. [57] observed engraftment and improved functional performance and improved survival after the injection of undifferentiated ES cells into the heart of mice with cardiomyopathy. Exploring ways to replace damaged or diseased hepatocytes, Yamamoto et al. [58] injected ES cells expressing EGFP under control of the albumin promoter, into the circulation of mice treated with carbon tetrachloride to injure endogenous hepatocytes. Some liver cells were subsequently found to express EGFP, suggesting that the ES cells had differentiated into hepatocytes.

ES cells might also be coaxed to differentiate into specific cell types in vitro and generate mature cells or tissues that might be transplanted for the treatment of disease. Thomson et al. [27, 55] suggested that the ability to proliferate indefinitely, to be genetically manipulated and to develop into all cell types in the body would make ES cells valuable therapeutically. For example, ES cells might be grown into large populations of undifferentiated cells and then coaxed to differentiate into cells useful for transplantation or tissue engineering. Klug et al. [59] cultured murine ES cells, transfected with the neomycin resistance gene under control of the α-cardiac myosin heavy-chain promoter, in the absence of LIF to induce differentiation into cardiomyocytes, injected the cardiomyocytes into the ventricular myocardium of dystrophic mice and found the cells to engraft and survive for as long as 7 weeks. Brustle et al. [60] found that murine ES cells cultured in the presence of basic fibroblast growth factor and platelet-derived growth factor differentiate into glial precursors that can myelinate the spinal cords of myelin-deficient rats upon transplantation. Basma et al. [61] performed sequential culture of human ES cells in fibroblast growth factor 2, human activin-A, hepatocyte growth factor and dexamethasone leading to the derivation of human hepatocytes that engrafted in the liver of mice and secreted albumin. Burt et al. [62] cultured ES cells in medium containing stem cell factor (SCF), IL-3, and IL-6 to encourage differentiation into hematopoietic precursors. The hematopoietic precursors rescued lethally irradiated mice when transplanted directly into the bone marrow cavity of the femurs whereas undifferentiated ES cells did not. This method allowed ES cells to be coaxed to differentiate into cell types for which the developmental cues necessary no longer exist.

Despite the successes mentioned above, the generation of functional, mature cells from ES cells in vitro has proven difficult. While most of the cell types in the body have been generated from ES cells, many of these cells exhibit impaired function. Lumelsky et al. [63] induced ES cells to differentiate into pancreatic

islet-like clusters that produce insulin in response to glucose in vitro. However, the cells produced only small amounts of insulin and did not reverse hyperglycemia in streptozotocin-induced diabetic mice. Furthermore, ES cells have yet to be grown in any culture system into an intact, transplantable organ.

3.3 Stem Cells and the Origin of Tumors

Pluripotent stem cells, which have a high proliferative capacity and the potential to form tumors as discussed above, exist in all mammals during development and frequently enter the circulation of mothers during pregnancy. Ariga et al. [64] found that all of 25 pregnant women with male fetuses had fetal cells in their circulation as detected by real-time PCR for the Y chromosome. Using PCR and Southern blotting, Bianchi et al. [65] detected male fetal cells in the maternal circulation 6 months to 27 years after parturition.

The fetal cells in the maternal circulation are likely pluripotent as they can contribute to maternal tissues [66]. Srivasta et al. [67] found fetal cells by fluorescent in situ hybridization for the Y chromosome in thyroid tissue in women who had previously given birth to male children. Khosrotehrani et al. [66] likewise detected male cells with the phenotype and appearance of hepatocytes in the liver of women who had previously given birth to male offspring.

Pluripotent stem cells from the fetus or intrinsic to the individual might form tumors. Cha et al. [68] found male cells in cervical cancer specimens of six out of eight women who had given birth to male offspring, suggesting that fetal cells can contribute to cancer in the mother. Stevens [69] found that spontaneous testicular teratomas in strain 129 mice arise from primordial germ cells in the fetal genital ridge that undergo aberrant development and become pluripotent stem cells. Stevens and Varnum [70] discovered that 50 % of LT strain mice develop ovarian teratomas due to parthenogenetic activation of their eggs.

The transplantation of ES cells and their derivatives may be hampered by the same propensity of these cells to form tumors. Behfar et al. [71] found that undifferentiated ES cells incapable of differentiating in response to TGF-β form teratomas in one-third of mice transplanted, whereas undifferentiated ES cells capable of responding to TGF-β do not. Arguing that teratoma formation occurs independent of TGF-β signaling, Nussbaum et al. [72] transplanted undifferentiated ES cells into the hearts of *nude* and syngeneic mice and found the formation of teratomas in 100 % of the animals. They similarly injected allogeneic undifferentiated ES cells into the hearts of Balb/c mice and found teratoma formation in all animals; however, these teratomas contained a robust inflammatory infiltrate around the third week, suggesting that rejection had been initiated.

Cells differentiated from ES cells can form teratomas. These teratomas might form because undifferentiated ES cells were not fully purged, or because the differentiated cells retained a capacity for pluripotency or some other property such as genetic instability. Dressel et al. [73] observed teratoma formation in 96 %

of mice following transplantation of undifferentiated ES cells into immunodeficient syngeneic and allogeneic mice, and in 95 % of mice following transplantation with neuronal cells differentiated from ES cells. The inoculum of differentiated cells did include a few cells expressing Oct3/4 and Ki67, as markers of undifferentiated cells: whether or not this small population of undifferentiated cells generated the tumors is, however, unknown.

Although tumors commonly form after transplantation of ES cells and cells differentiated from them, teratomas and embryonic carcinomas are relatively infrequent in the natural setting. Presumably the fetus and the mother have powerful surveillance and control that prevent tumor formation. Why these mechanisms fail after transplantation is an important question.

3.4 Innate Immunity as a Barrier to Tumor Formation

Innate immunity may prevent formation of tumors by pluripotent stem cells. While innate immunity provides the first line of defense against infection, it also may protect against tumors.

3.4.1 The Complement System

One component of the innate immune system that might rapidly recognize and destroy tumor cells is the complement cascade. The complement cascade consists of a series of more than 30 soluble and membrane-bound proteins. These proteins are activated by one of three pathways: repetitive polysaccharide structures conserved on many microbes (lectin pathway), other conserved surface structures of viruses and microbes (alternative pathway) and membrane bound antibodies (classical pathway). The three complement pathways converge on the terminal complement complex which can lead to cellular lysis. Other additional mechanisms of protection include opsonization or coating of a pathogen, targeting it for phagocytosis or other mechanisms of induced cell death. The by-products of the complement cascade can also generate inflammation and recruit inflammatory cells such as neutrophils.

In the absence of effective control of the complement cascade most fetal cells are rapidly targeted for destruction. In rodents, the membrane bound complement regulatory protein *Crry* prevents the deposition of C3 and C4 components of complement on cells. Xu et al. [74] showed that rat fetal cells are killed by the complement cascade in the absence of *Crry*. The authors performed $Crry^{+/-}$ *x* $Crry^{+/-}$ matings and observed no $Crry^{-/-}$ offspring in over 245 live births. However, when $Crry^{+/-}$ *x* $Crry^{+/-}$ matings were carried out on a complement deficient background ($C3^{-/-}$) the expected number of $Crry^{-/-}$ offspring were

observed, illustrating the importance of control of the complement cascade for normal reproduction.

Although pluripotent stem cells clearly cross the maternal-fetal interface, tumor formation from these cells is rare. Absence of tumors in mother and fetus might be explained if pluripotent cells were susceptible to complement and differentiated cells were not. Consistent with this concept, Koch et al. [75] observed that undifferentiated ES cells are exquisitely sensitive to complement (more than 70 % of undifferentiated ES cells were killed) while cardiomyocytes differentiated from the ES cells are not (less than 20 % killed). Also consistent with this concept, undifferentiated ES cells formed teratomas more quickly in complement-deficient than in complement-sufficient mice. Since the concentration of complement in blood exceeds the concentration in extra-vascular spaces, this control might explain how small numbers of stem cells persist in extra-vascular sites without formation of tumors.

3.5 Alloimmunity and ES Cells

The most significant barrier to the engraftment and function of ES cells and their derivatives is alloimmunity. With the evolution of multicellular organisms came the need to differentiate self from non-self in order to eliminate foreign micro-organisms. Differentiation and elimination of foreign cells can readily be accomplished due to the large number of foreign antigens encountered, giving ample targets for the immune system. A more daunting challenge is the recognition and elimination of allogeneic cells which closely resemble the host.

The main determinants of alloimmunity and the rejection of transplants are major and mH antigens. The MHC encodes a series of proteins that present foreign and self peptides synthesized within the presenting cell (MHC class I) or foreign and self peptides produced from proteolysis of proteins phagocytosed by the cell (MHC class II). The peptides presented by the MHC molecules are recognized by T cells. The MHC molecules are polymorphic and, when differing from the host's own complement of MHC molecules, stimulate a vigorous immune response leading to graft rejection. mH antigens are foreign peptides presented in the context of the host's MHC molecules and can initiate a similarly vigorous immune response and graft rejection when differing from the host's own repertoire of self peptides.

In humans, all grafts bearing foreign MHC antigens are rejected in the absence of immunosuppression. When MHC antigens are matched some, but not all, grafts reject owing to incompatibility of foreign mH antigens. These "rules" of histocompatibility apply to nearly every type of tissue and tumor studied [4] and a similar fate would be expected to await grafts of ES cells or their derivatives. However, in some instances, semi-allogeneic and fully-allogeneic ES cells are not destroyed like other cells and instead grow into teratomas [76]. Below we discuss

various mechanisms that might explain the absence of rejection of ES cells and their derivatives.

3.5.1 Immune Tolerance

Immune tolerance can be defined as antigen-specific immune non-responsiveness. The concept of tolerance explains why immunity can be raised to foreign cells and substances but not to autologous cells and self components. Since all histo-incompatible cell and tissue transplants are rejected (by cellular immunity), absence of rejection may reflect T cell tolerance. T cell tolerance develops either in the thymus (central tolerance) by deletion of potentially self-reactive T cells, in the peripheral lymphoid organs (peripheral tolerance) or by the generation of regulatory T cells. Tolerance has been successfully induced to allografts in mouse models but, unfortunately, can not be simply and reliably induced for human allografts and tolerance does not occur spontaneously in a mother to the fetus [2].

Undifferentiated ES cells were found to induce tolerance in fully MHC-mismatched recipients in at least one model system. Fandrich et al. [77] injected pre-implantation ES cells from rats into the portal veins of fully MHC-mismatched rats and found the allogeneic cells engrafted for longer than 150 days without signs of rejection. Remarkably, subsequent cardiac allografts from the same allogeneic strain were spontaneously accepted. The authors hypothesized that the pluripotent stem cells induced chimerism, which in turn led to central tolerance. Such a result would be consistent with the observation that induction of mixed hematopoietic chimerism, in which both donor and recipient bone marrow stem cells are engrafted, induces tolerance to allografts [78, 79].

The ability of ES cells to induce tolerance has been called into question by others. Magliocca et al. [80] injected murine ES cells into the portal veins of allogeneic mice and found no evidence of chimerism or the ability to promote cardiac allograft survival. We also were unable to induce tolerance to murine skin allografts by intraportal injection of undifferentiated ES cells (unpublished observation).

Differences between the findings of Fandrich et al. [77] and findings of others might be explained in several ways. First, the cells used by Fandrich et al. [77], which are referred to as “ES cell-like” cells, may not have been ES cells but some other stem cell population with different characteristics. Consistent with this possibility, the ES cell-like cells expressed MHC class II molecules, which are not typically expressed by ES cells, and did not form teratomas in tolerant hosts.

While doubt has been cast on the ability of ES cells to induce tolerance, derivatives of ES cells may prove useful to this end. ES cells successfully coaxed to differentiate into hematopoietic stem cells could be used to induce mixed chimerism in the recipient prior to transplantation of the cell or tissue allograft, potentially leading to long-term survival [81]. The use of this differentiated population of cells might decrease the risk of teratoma formation compared to

transplants of undifferentiated ES cells. Burt et al. [62] cultured undifferentiated ES cells in methylcellulose containing SCF, IL-3, and IL-6 causing them to differentiate into c-kit$^+$/CD45$^+$ hematopoietic stem cells. When administered to lethally irradiated mice, these cells induced mixed chimerism with tolerance to both donor and recipient cells but left intact immune responses to third party MHC-mismatched cells. Bonde et al. [82] coaxed undifferentiated murine ES cells to differentiate into CD45$^+$ hematopoietic stem cells by transfecting them with the hematopoietic transcription factor HoxB4. Administration of these cells to sublethally irradiated MHC-mismatched recipient mice led to enduring mixed chimerism and tolerance. Treated mice thus accepted cardiac allografts from the donor strain but rejected allografts from other strains.

3.5.2 MHC Expression by ES Cells

Absence of immunity to ES cells could reflect failure of the immune system to recognize them as foreign. Alloimmune responses are mainly directed against MHC molecules expressed by foreign cells. Decreased or absent expression of foreign MHC molecules by undifferentiated ES cells could, therefore, make them difficult to detect by the recipient's immune system, preventing the initiation of an alloimmune response.

The expression of MHC molecules has been shown to be decreased on undifferentiated ES cells compared to differentiated cells. Tian et al. [83] characterized the expression of the immunoglobulin superfamily of cell adhesion molecules, which includes MHC class I and II molecules, on murine ES cells and found no expression of MHC class I or MHC class II molecules. Neither did expression of MHC class I and MHC class II molecules increase following exposure of undifferentiated cells to the inflammatory cytokines IFNγ or TNFα for 48 h. Drukker et al. [84] found that three human ES cell lines express very low levels of MHC class I molecules and that the expression of MHC class I molecules significantly increased with differentiation or with exposure to IFNγ. None of the cell lines studied, however, expressed MHC class II molecules or the non-classical MHC molecule HLA-G. Lampton et al. [85] studied the MHC expression of murine ES cells derived from fertilized embryos compared to that of murine ES cells derived from parthenogenesis and found that the mRNA for MHC class I antigens, as well as that for antigen processing and chaperone proteins, was expressed at similarly low levels and both the mRNA and protein expression was significantly increased following exposure to IFNγ.

While ES cells express low levels of MHC class I and no MHC class II molecules, almost all differentiated cells express MHC class I proteins. However, Tian et al. [83] reported that cells differentiated from murine ES cells following culture of the cells in the absence of LIF for 2 weeks failed to significantly upregulate the expression of MHC class I molecules compared to control undifferentiated ES cells. However, exposure of the differentiated cells to IFNγ increased the

expression of MHC class I molecules fivefold. These results should be interpreted with caution, as the differentiated cell population studied was heterogeneous and the degree of differentiation of the cells was never determined. Boyd and Wood [86] studied the expression of MHC class I and MHC class II molecules on undifferentiated ES cells and insulin producing cell clusters derived from them. Undifferentiated ES cells and immature insulin producing cell clusters expressed low levels of MHC class I molecules and no MHC class II molecules in contrast to mature insulin-producing cell clusters which expressed MHC class I molecules and would express low levels of MHC class II molecules following stimulation with IFNγ.

If the low level expression of MHC molecules by ES cells protects the cells from attack by T cells, it may make them more susceptible to attack by natural killer (NK) cells. NK cells attack and kill cells lacking or deficient in MHC class I because MHC molecules provide an inhibitory signal to the NK cells. Thus, low or absent expression of MHC molecules by ES cells might render them susceptible to killing by NK cells. However, Drukker et al. [84] found that undifferentiated human ES cells were not killed by human NK cells despite low levels of MHC expression. Similar results were seen with early differentiated cells in the form of embryoid bodies.

Others, however, do find that ES cells are susceptible to NK cell-mediated cell death. Dressel et al. [87] reported that pluripotent stem cells including multipotent adult germline stem cells as well as ES cells were susceptible to NK cell-mediated killing using an in vitro assay of lysis. They attributed this susceptibility to the expression of ligands for NKG2D, an activating receptor on NK cells, found on the majority of pluripotent stem cells tested as well as the ability of soluble NKG2D added to cultures to inhibit the cytotoxicity. These authors also found activated NK cells slowed (but do not fully prevent) the formation of teratomas.

3.5.3 Immune Privilege and Site-Specific Immunosuppression by ES Cells

Although the mammalian fetus is semi-allogeneic with respect to the mother and hence should be subject to immune attack, such attack does not normally occur. Failure of the immune system of the mother to attack the fetus does not reflect either immunological tolerance or immunological ignorance since repeated pregnancies both sensitize the mother and heighten the success of further pregnancies with the same father [2]. Failure of the mother to 'reject' the fetus in the face of potent immunity to paternal antigens could reflect local control of immunity or shielding from injury. For want of a more precise term we call this condition "site-specific immunosuppression".

ES cells, or their derivatives like a fetus, might possess unique characteristics that suppress immune responses. To determine whether the host's immune system

recognizes and mounts an attack against ES cells, Grinnemo et al. [88] transplanted human ES cells into immunologically competent mice and observed a robust infiltrate of T cells and macrophages within 3 days, similar to what was observed following the transplantation of human fibroblasts. The authors also found that human ES cells stimulated T cell proliferation in mixed leukocyte cultures at the same levels as human fibroblasts and concluded that ES cells were recognized and rejected similar to transplants of differentiated cells. It should be noted, however, that the experimental system used was xenogeneic and may not translate to allogeneic models.

It has also been suggested that undifferentiated ES cells are recognized and rejected like differentiated allogeneic cells. Nussbaum et al. [72] transplanted undifferentiated murine ES cells into the hearts of allogeneic mice and found that the cells form teratomas; however, the teratomas began to show a significant inflammatory infiltrate by 3–4 weeks and were almost completely replaced by inflammatory cells after 5 weeks. This suggests that the ES cells were recognized and rejected by the host immune system. The rejection of the teratomas in this model was preceded by upregulation of MHC class I and MHC class II molecules, suggesting that it may have been stimulated by differentiated cells and not the undifferentiated ES cells initially transplanted. Supporting this possibility, Swijnenburg et al. [89] found that undifferentiated murine ES cells transplanted into the hearts of allogeneic mice generated minimal inflammatory infiltrate. However, the inflammatory infiltrate became more robust at 4–8 weeks with subsequent rejection of the graft as teratomas formed and the undifferentiated ES cells differentiated.

While the allogeneic immune system appears able to recognize and, in some cases, to reject transplants of ES cells, ES cells can sometimes modulate and evade allogeneic immune responses. Bonde and Zavazava [90] found that undifferentiated murine ES cells suppress T cell proliferation and killing in in vitro assays and that intravenous injection of undifferentiated ES cells induces low levels of chimerism leading to suppression of allogeneic immune responses. The authors attributed their results to the expression of FasL by ES cells, which induces apoptosis in cells expressing the Fas receptor, leading to deletion of alloreactive T cells. Koch et al. [76] transplanted undifferentiated murine ES cells in allogeneic and semi-allogeneic hosts and found that teratomas form in a dose-dependent manner. Mice receiving allogeneic or semi-allogeneic ES cells rejected subsequent skin grafts MHC matched to the ES cells more quickly than mice that did not receive ES cells suggesting that the allogeneic or semi-allogeneic ES cells stimulated immunity without culminating in rejection. These results were ascribed to site-specific immunosuppression by ES cells due to the secretion of TGF-β. Wu et al. [91] transplanted murine ES cells expressing the MHC molecule H-2K^{b} into the kidney capsule of BM3-*Rag*$^{-/-}$ mice with monoclonal CD8^{+} T cells specific for H-2K^{b}. The T cells did not become activated and did not infiltrate the ES cell graft. The lack of recognition of the T cells could be overcome by transferring APCs matched to the ES cells or transferring previously activated T cells which could reject the grafts.

Whether cells differentiated from ES cells also suppress immune responses against them is unknown. Robertson et al. [92] differentiated murine ES cells into embryoid bodies and transplanted the embryoid body cells into mice differing in only mH antigens. The transplants were rejected. However, tolerance could be induced using minimal induction protocols with anti-CD4 and anti-CD8 monoclonal antibodies, suggesting the differentiated cells exerted at least some of the immunomodulatory properties of the undifferentiated ES cells. The authors attributed the ease of tolerance induction in this model to the secretion of TGF-β2 by the ES cells and cells differentiated from them.

3.6 Conclusions

The potential application of ES cells for the future treatment of human disease requires a detailed understanding of complications ensuing from interaction with the immune system. ES cells provoke less immunity than differentiated cells and the mechanisms may be multiple. Absence of immunity, particularly site-specific suppression of immunity, may protect pluripotent stem cells and the entire fetus from the immune system of the mother. The immune suppression or privilege of ES cells might be exploited when applying ES cell technology for the treatment of human disease but may also protect the 'privileged' tumor from surveillance.

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Chapter 4
The Role of NK Cells and T Cells in the Rejection of Embryonic Stem Cell-Derived Tissues

Bob Miyake and Nicholas Zavazava

Abstract Embryonic stem (ES) cells provide a unique opportunity for the establishment of new cell-based therapies that could benefit human health in the future. However, before that step can be taken, we need to better understand how to manipulate the differentiation of stem cells into cell lineages of interest and to define the immunological characteristics of these cells in the context of allogeneic transplantation. Here, we will discuss the interaction of ES cell progenitors with alloreactive T cells and NK cells and discuss how low MHC expression by ES cell derivatives may be exploited for therapies in humans.

4.1 Introduction

Allogeneic tissues and organs are rejected post transplantation unless preconditioning regimens and immunosuppression are sufficient to counteract immunological rejection. Alternatively, graft-versus-host-disease (GvHD) can also develop secondary to transplantation of immune competent effector T cells in the donor graft. These immune reactions are based on histocompatibility antigens and minor histocompatibility (mH) disparities between donor and recipient. Recognition of these antigens as foreign, in either the graft or the recipient can lead to detrimental immunological reactions. In general, there are three classes of antigens that impact immune recognition: major histocompatibility complex (MHC) antigens, mH antigens, and ABO blood group antigens [1, 2]. Highly vascularized organ grafts may undergo rejection mediated by ABO incompatibility. However, ABO incompatibility does not appear to play a

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significant role in allogeneic hematopoietic cell transplantation [1]. Here, we will focus on MHC, mH antigens, and embryonic stem (ES) cells and their derivatives.

Previous work on mouse ES cells in our laboratory [3] and on human ES cells by Drukker [4] have shown that these cells express low levels of MHC class I and virtually no MHC class II antigens. When transplanted across MHC barriers, low level expression of MHC class I may limit detection by cytotoxic T cells (CTLs) but could trigger rejection by NK cells. As one might infer, this contrast of antigen/ligand recognition patterns between T and NK cells creates a potential dichotomy in transplanting ES cells and their derivatives into allogeneic recipients.

It is unclear why ES cells do not express robust levels of MHC antigens. After stimulation with IFN-γ, we have reported upregulation of MHC class I antigens by both mouse and human ES cell derivatives [5, 6]. However, we detected no upregulation of class II antigens. Others have recently reported that lack of class II upregulation is due to lack of expression of CIITA [7]. If confirmed, it would appear that the lack of robust immunogenicity of ES cell-derived cells is due to poor expression of transcription factors that regulate MHC expression in these cells. We, however, recently reported that ES cell-derived hematopoietic cells eventually express high levels of both class I and class II antigens after 4–8 weeks post transplantation, suggesting that as the cells mature, they behave like adult cells [5]. Given these interesting findings, we will further discuss the importance of MHC antigens in transplant immunology and the interaction of T lymphocytes and NK cells with ES cells and their derivatives.

4.2 Expression of MHC Antigens by ES Cells and Their Derivatives

To fully appreciate the immunological hurdles involved in transplanting allogeneic cells, in particular ES cell-derived cells, it is important to understand the role of MHC antigens in stem cell biology. The MHC, otherwise known as human leukocyte antigens (HLA) in humans, contains over 200 genes on chromosome 6, of which more than 40 have been found to encode leukocyte-associated antigens [8]. The protein products of these polymorphic MHC genes can be generally grouped into two distinct classes: MHC class I and MHC class II. Crystographic structural analysis of MHC molecules has shown that class I determinants are heterodimers consisting of an α-polypeptide subunit (44 kD) non-covalently linked to a β2-microglobulin (12 kD) light chain [9] (HLA-A, HLA-B and HLA-C). The β2-microglobulin chain is encoded by a gene on chromosome 15 [8]. Endogenous peptide fragments are presented within the peptide binding groove of MHC class I for recognition by $CD8^+$ T cells. On the other hand, class II antigens are heterodimers consisting of α and β polypeptide subunits (HLA-DP, HLA-DQ, and HLA-DR) and bind exogenous peptides that have been endocytosed and processed for presentation to $CD4^+$ T cells. It is the bound peptide that defines the specificity of T cells recognizing these molecules.

MHC class I antigens are expressed by most nucleated somatic cells. However, the level of expression is variable and depends on the particular tissue involved [8]. In contrast, MHC class II antigens are restricted to antigen-presenting cells such as macrophages, dendritic cells, B cells, activated endothelial, and thymic epithelial cells [8]. In humans, activated T cells also express MHC class II antigens. Both classes of antigens can elicit immune responses in an allogeneic setting and pose a substantial barrier to transplantation. In fact, the amount of antigenic disparity of MHC antigens between donor and recipient is a key determinant in transplant outcome. When evaluating bone marrow transplantation data and comparing different HLA loci, disparities in HLA-A, -B, -C, and -DRB1 appear to be consistently associated with adverse outcomes [10].

Thus, significant work has been invested in understanding the clinical outcomes of HLA mismatch donors and their recipients. Baxter-Lowe et al. [11] evaluated HLA-A mismatches in adult transplant patients under the National Marrow Donor Program and found six mismatches with deleterious effects. However, to detect an effect on survival of any one of the six mismatches and achieve 80 % power, total transplant populations of 11,000 to over 1 million donor-recipient pairs were required and depended on the specific HLA-A disparity involved. It should be noted, however, that HLA haplotype mismatches can be advantageous in certain settings of leukemia, where the graft-versus-leukemia (GvL) effect can help to eliminate residual disease [12]. It seems clear that MHC (or HLA) mismatches can be complex in the clinical setting and that this complexity results from the extraordinary diversity within specific HLA molecules.

However, this complexity and the relative difficulty in identifying optimal HLA matches is what generates significant interest in investigating ES cells (and their derivatives) in transplant medicine. Based on our understanding of MHC antigens, investigators have looked into ES cells and levels of MHC expression. Data thus far have showed intriguing results. In human ES cells, Drukker et al. [4] were able to show low expression levels of MHC class I and virtually no MHC class II expression in H9 and H13 cell lines. Furthermore, in vitro and in vivo differentiation of these ES cells did not result in a significant increase in MHC class I expression. Similarly, mouse ES cells have been shown to have low-level expression of MHC class I and no MHC class II expression [13, 14] (Fig. 4.1).

It appears though that there are some differences between different progenitor cell types. For example, low MHC levels have been reported on hematopoietic cells, cardiomyocytes, and neuronal progenitors derived from ES cells. However, data from Preynat-Seauve et al. [15] appear to show rapid rejection of ES cell-derived neuronal cells, which is in sharp contrast to hematopoietic cell progenitors in both the mouse and in humans [3, 5, 6]. So far, no generalized statements can be made because of these unique tissue-specific differences.

Although low-level MHC expression would appear advantageous for the minimization of ES cell interaction with T lymphocytes, it is unclear whether this remains the case post transplantation in light of numerous serum cytokines and growth factors that might influence antigenicity. The potential inflammatory environment encountered by ES cells after transplantation may affect surface

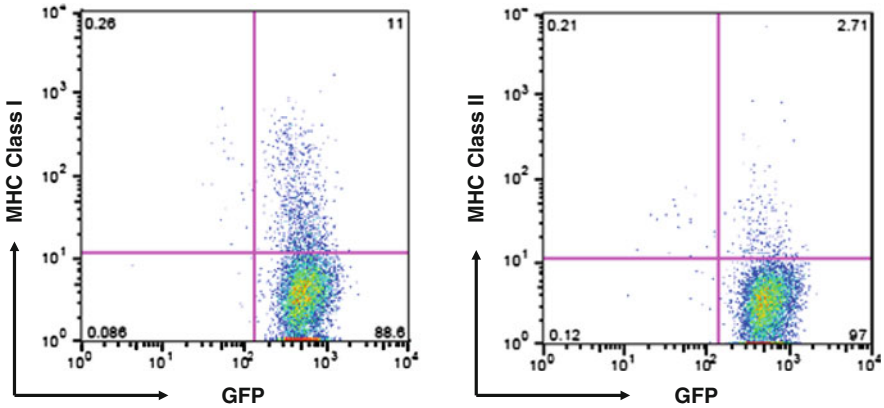

Fig. 4.1 ES cells express low levels of MHC class I and are class II negative. Hematopoietic progenitor cells were derived from (BALB/c x 129 SvJ) ES cells that were transduced with GFP. The cells were stained with PE-conjugated antibodies against MHC class I and class II antigens and analyzed by flow cytometry

expression of MHC antigens. Indeed, our most recent data show poor susceptibility of ES cell-derived hematopoietic cell progenitors to NK cells in vitro. However, when transplanted in NK cell-rich RAG2$^{-/-}$ mice, these progenitors were deleted [16]. So far it is unclear why there is this discrepancy. This finding serves as a reminder that in vitro observations may not be a true reflection of what happens in vivo. We therefore need more in vivo data to improve our understanding of stem cell biology. For example, earlier observations appeared to suggest that ES cell-derived progenitor cells are immune privileged [17]. In light of our data, however, it would appear that ES cell-derived cells show low immunogenicity, rather than immune privilege. Indeed, in kinetic studies carried out in our lab, MHC expression was monitored over 100 days in ES cell-derived hematopoietic progenitor cells post transplantation. We found that class I antigen was gradually upregulated and had normalized to that of bone marrow cells by 60 days post transplantation [5]. MHC class II expression followed a similar trend but was only positive in a subpopulation of our cells. This eventual increase in MHC expression could substantially influence the ability of ES cell-derived progenitors to incite allorecognition and rejection.

4.3 Interaction of ES Cells and Their Progenitors with T Cells

Data on the propensity of ES cells and their derivatives to be recognized by T cells are very limited. Initial studies concentrated on ES cells themselves, although it is ultimately tissues differentiated from ES cells that are the more relevant cell

population that will be clinically applied. For example, we observed that when ES cells were used as stimulator cells in a mixed leukocyte reaction, they failed completely to stimulate T cells [18]. This finding led us to further investigate possible reasons for this. Apart from low MHC expression, we observed that ES cells express FasL which induced apoptosis in T cells. We further examined whether ES cells were susceptible to NK and cytotoxic T lymphocyte (CTL) killing. Activated, but not naïve, NK cells modestly lysed ES cells [13]. However, CTLs did not lyse ES cells, a consequence of the low MHC expression but also possible ES cell-induced apoptosis.

More recently, Fairchild and colleagues [19] showed that mH antigen differences between ES cell derivatives and recipients triggered immune rejection. However, only embryoid bodies were used in those studies, which are mixtures of all three germ layer products with some undifferentiated cells. It would be interesting to see whether this is the case with purified lineage specific cells. More interestingly, they did show that minimal conditioning of recipients was sufficient to induce tolerance. In contrast, in our own published data, we showed that purified ES cell-derived hematopoietic progenitor cells induced mixed chimerism which protected donor specific, but not third-party cardiac allografts from rejection [5]. We further showed that the progenitors populated the thymus, suggesting that clonal deletion of alloreactive T cells might be the mechanism by which tolerance was induced in this model. Another interesting aspect is that ES cell-derived progenitor cells may impact tolerance by producing immunosuppressive cytokines such as TGF-β [20]. However, more definitive experiments need to be pursued.

We have also determined the immunogenicity of ES cell-derived cardiomyocytes and that of ES cell-derived definitive endodermal cells. None of these cells expressed either class I or class II antigens in vitro. Studies on how these cells behave in vivo remain to be seen. It is possible that serum cytokines could stimulate these cells to express MHC antigens, making them susceptible to alloreactive CTLs.

4.4 Impact of NK Cells on Engraftment of ES Cell Derivatives

The low expression of MHC class I antigens by ES cell derivatives could make them vulnerable for NK cell recognition and lysis. While poor MHC expression is an advantage for avoiding T cell recognition, cells that express MHC antigens only weakly, such as tumor cells, can be lysed by NK cells instead.

There are several receptors expressed by NK cells that mediate their function. Natural cytotoxic receptors include NKp30, NKp44, and NKp46; NK cell activating receptors include NKG2D, DNAM-1, and CD16 [21]. NK cells also possess inhibitory receptors known as killer cell immunoglobulin-like receptors (KIRs) that recognize autologous (inhibiting) ligands. Recognition of these ligands will

inhibit NK cell cytotoxicity and prevent reactivity toward self [22]. By recognizing "missing self", NK cells will lyse allogeneic targets that do not express inhibitory ligands [22]. Interestingly, recognition of missing self is evident even in haploidentical hematopoietic transplantation. In studies where donor NK cells express a KIR for the absent HLA class I, these NK cells will sense the "missing expression of self" and mediate an alloreaction. For example, Ruggeri et al. [22] proposed that leukemia relapse might be decreased by effective donor-vs-recipient alloreactive NK cells with molecular high-resolution HLA typing and KIR genotyping of donors [23], a strategy being pursued in several clinical trials.

Our most recent data on ES cell-derived hematopoietic progenitor cells indicate that these cells express the NK cell ligand H60 at high levels [16]. In addition, when the cells are transplanted, they upregulate Rae1 and Mult1 making them highly susceptible to NK cell killing. Interestingly, this may not be the case for non-hematopoietic derivatives. For example, when we examined ES cell-derived definitive endodermal cells, we observed that these cells do not express H60 and are not recognized by NK cells. Further insight into NK cell function and their ability to eliminate unwanted hematopoietic cells was recently provided by Dong et al. [24]. By using triple-knockout mice (SAP-, EAT-2-, and ERT-) to affect SAP-related adaptor and SLAM receptor signaling, they were able to show defective NK cell activity toward malignant hematopoietic cells. Based on their findings, they proposed that loss of MHC class I on hematopoietic cells would initiate NK cell cytotoxicity via SLAM-SAP pathways [24].

Thus, NK cell activity poses another significant barrier to hematopoietic stem cell transplantation. However, every ES cell-derived tissue or cell type will need to be evaluated for the expression of NK cell ligands. Without the expression of NK cell ligands, such tissues are less likely to be recognized by NK cells. We have also reported that deletion of NK cells using an anti-NK1.1 antibody promotes the engraftment of ES cell-derived hematopoietic progenitor cells. Unlike solid organ transplantation, where the role of NK cells in organ rejection is considered minimal, hematopoietic cells are excellent targets. Thus, a possible transplantation strategy could be to delete NK cells pre- and post transplantation. However, more data are required to provide insight into other non-hematopoietic ES cell derivatives.

4.5 Summary

ES cells and their derivatives have tremendous potential in the treatment of chronic and debilitating diseases. However, as we have discussed, our full understanding of these cells in allogeneic transplantation is limited. We know that recognition and targeting by CTLs and NK cells is significant when transplanting across MHC barriers. In the case of ES cells and their derivatives, data support the fact that these cells have low immunogenicity in spite of MHC disparity. Nonetheless, further characterization of in vitro and in vivo responses of CTLs and NK cells toward ES cell derivatives needs to be investigated. Accordingly, the behavior of ES cells and

their derivatives may be different in vitro and in vivo. It is possible that lineage-specific changes occurring post transplantation also influence the immunogenicity of ES cell derivatives in ways other than MHC expression. Such differences will certainly impact the clinical applicability of specific ES cell derivatives.

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Chapter 5
Mitigating the Risk of Immunogenicity in the Pursuit of Induced Pluripotency

Paul J. Fairchild and Naoki Ichiryu

Abstract The advent of induced pluripotent stem (iPS) cells represents a significant milestone in the field of regenerative medicine. While the first derivation of human embryonic stem (hES) cells 8 years earlier, had made pluripotency accessible in vitro for the first time, iPS cells offered the elixir of personalised pluripotency by facilitating the generation of autologous lines, tailored to the needs of the individual. Importantly, an autologous source of iPS cells promised to circumvent the immunological barriers that have threatened to undermine the translation of cell therapies to the clinic. Nevertheless, quite apart from the practical and economic constraints of personalised medicines that may prohibit their widespread implementation, recent studies have questioned whether tissues derived from iPS cells in an autologous fashion will be ignored by the immune system of the recipient. Indeed, the up-regulation of developmental antigens upon reprogramming and their persistent expression during differentiation may render such tissues vulnerable to rejection. Here, we assess the likely impact that such findings will have on the clinical application of induced pluripotency.

5.1 Introduction

While increased life expectancy throughout the developed world remains one of the most tangible benefits of advances in medical science, the unanticipated consequences for healthcare in the twenty-first century are no less profound. Indeed, the rising incidence of chronic and degenerative diseases due to the ageing

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nature of the population, threatens to consume an unsustainable proportion of the healthcare budgets of developed nations and eclipse the needs of the developing world, for which infectious disease remains a far greater priority. There is, therefore, a significant medical imperative to develop effective treatments for degenerative diseases that go beyond the alleviation of symptoms to the restoration of the function of tissues compromised through the natural process of ageing. It is this mandate for which the properties of pluripotency offer a potential solution by providing a novel source of cell types of therapeutic value, that may one day replace endogenous cells lost to disease or advancing age.

Against this backdrop, the first derivation of embryonic stem (ES) cell lines from supernumerary human blastocysts [1], heralded a new era of regenerative medicine by capturing the elusive state of pluripotency and making it accessible in vitro for both academic study and therapeutic application. Indeed, the capacity for indefinite self-renewal of hES cells, coupled with their propensity for directed differentiation along defined lineage pathways, has provided an attractive strategy to address the needs of an ageing population, their capacity for scalability providing the potential to meet the rapidly increasing demand for cell replacement therapies (CRT). Nevertheless, while our understanding of pluripotency and its ordered perturbation during differentiation has increased substantially over the past decade, equivalent advances in related fields have failed to keep pace, of which the immunology of allograft rejection is especially relevant. Indeed, early speculation that tissues differentiated from ES cells may not be rejected by allogeneic recipients due to their low expression of MHC determinants and lack of endogenous dendritic cells (DC), has consistently failed to find support from various mouse models of CRT [2, 3], emphasising the urgent need to find ways of further reducing the immunogenicity of replacement tissues. While somatic cell nuclear transfer offers a potential source of cloned blastocysts from which ES cells may be derived that share their genotype with the nuclear donor [4], the practicalities and ethical complexities of therapeutic cloning have posed insurmountable obstacles to its routine use [5, 6]. The first formal demonstration in 2006 that somatic cells could be reprogrammed to pluripotency by the introduction of as few as four transcription factors [7], therefore, proved to be revolutionary, providing a plausible alternative route to personalised therapies, capable of circumventing the impasse created by both ethical and immunological sensitivities. Given that 6 years have now passed since the publication of this seminal work, it is perhaps legitimate to ask to what extent the promise of induced pluripotency has lived up to these early expectations.

5.2 Induced Pluripotency: A New Chapter in the History of Regenerative Medicine

Few topics in the biomedical sciences have gathered momentum in quite the same way as induced pluripotency. Early reports of reprogramming demonstrated that retroviral transduction of mouse embryonic fibroblasts with the transcription

factors Oct4, Sox2, Klf4 and c-Myc produced self-renewing stem cell lines capable of generating tissues derived from each of the three embryonic germ layers, but stopped short of demonstrating their capacity to give rise to viable progeny [7]. Nevertheless, the subsequent use of tetraploid complementation to produce mice wholly derived from iPS cells [8, 9], provided unequivocal proof of their pluripotency and catalysed rapid advances in this nascent field. The application of the same protocols to cells derived from rats [10], rhesus macaques [11] and humans [12–14] confirmed reprogramming to be universally applicable across species: indeed, during the past few years, iPS cells have been generated from a broad range of mammalian species, even the white rhino, which is currently endangered [15]. Furthermore, while significant differences have been reported between cell types in terms of the efficiency of reprogramming, none has so far failed to give rise to iPS cells, making the technology especially versatile and greatly increasing its likely future impact on biomedical science. While early studies relied heavily on the use of retroviral transduction that undermined any downstream clinical application, recent efforts have focussed on the use of non-integrating vectors [16], micro-RNAs [17], the delivery of recombinant transcription factors [18, 19] or their replacement with small molecules capable of mimicking their mode of action [20, 21]. Although such creative approaches to reprogramming have enjoyed varying degrees of success, the stability of the pluripotent states generated have yet to be rigorously compared. Nevertheless, such results suggest that it is merely a matter of time before reproducible protocols are developed that are clinically compliant and that may be universally adopted to introduce a much-needed degree of standardisation to such a rapidly evolving field.

Although such an endeavour has yet to reach a clear consensus, induced pluripotency has begun to yield insights into a variety of disease states by providing in vitro correlates of human disease which are particularly welcome in the case of diseases for which animal models are unavailable or inadequately recapitulate their human counterparts [22]. By generating iPS cell lines from small tissue biopsies taken from individuals diagnosed with conditions whose aetiology is only poorly understood, it has proven possible to immortalise the disease-associated genotype in a population of self-renewing stem cells whose pluripotency may permit their subsequent differentiation into the very cell types affected by the disease. Using this approach, it has already proven feasible to model not only a broad range of simple monogenic disease states [23], but rather more complex traits, such as schizophrenia [24], providing valuable insights into the molecular and cellular basis of disease progression, while facilitating the screening of libraries of compounds for potential new drugs, capable of ameliorating the symptoms of disease.

Although the use of iPS cells as a source of cell types and tissues for administration to patients currently awaits the development of clinically compatible protocols for their derivation, their unique properties undoubtedly augur well for their future application to regenerative medicine. It has, for instance, been demonstrated that the age of the donor from which the biopsy was taken, poses no inherent obstacle

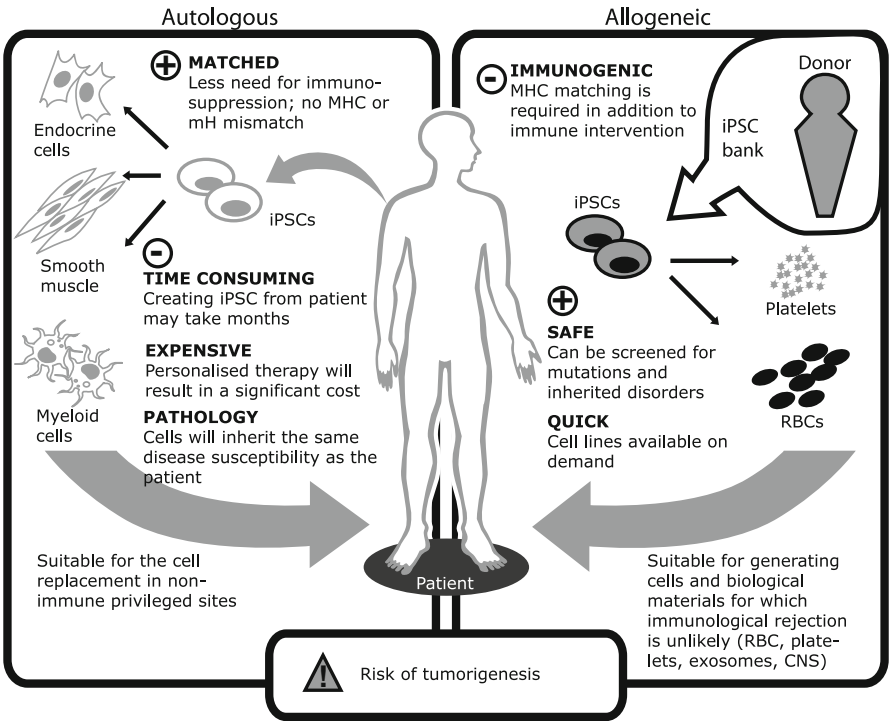

Fig. 5.1 The debate surrounding the use of allogeneic versus autologous sources of cell types for CRT remains unresolved since either source carries its own advantages and disadvantages. Importantly, the advent of iPS cell technology has yet to provide a clear consensus as to which route to the clinic is most likely to prove feasible

to the success of reprogramming, a principle epitomised by the successful generation of iPS cells with demonstrable pluripotency from an 82-year-old donor [25]. Such considerations are, of course, relevant to the development of strategies that might one day treat chronic and degenerative disease of an ageing population. Second, like ES cells, iPS cells are relatively tractable candidates for genetic modification, providing opportunities for the ex vivo correction of genetic defects that drive the disease phenotype. Hanna and colleagues have, for example, performed a proof of principle study in a mutant strain of mice modelling human sickle cell anaemia. By replacing the mutant gene with its wild type counterpart in iPS cells derived from this strain, they were able to rescue the phenotype by administration of hematopoietic progenitors differentiated from them [26]. Although translation to human cells has not been straightforward, a similar approach has successfully replaced the mutant gene in iPS cells derived from a patient with Fanconi anaemia and demonstrated that hematopoietic progenitor cells differentiated from them, are able to function normally [27]. Furthermore, in a recent study, An and colleagues established iPS cell lines from patients suffering from Huntington's disease and employed homologous recombination to correct the CAG expansion in the huntingtin gene that drives

widespread cell death in the striatum of patients [28]. While such technological achievements, coupled with the ability to generate iPS cells in a fully autologous manner, appear to offer a well-defined pathway to the clinic, they have, nevertheless, served to fuel the ongoing debate over the relative merits of autologous versus allogeneic sources of stem cells (Fig. 5.1), an argument that recent studies have revealed is rather more finely balanced from an immunological perspective than has previously been appreciated.

5.3 The Case for Allogeneic iPS Cells

5.3.1 An Economic Reality Check

While aspirations to harness the potential of induced pluripotency in a fully autologous manner remain buoyant in some quarters, few could doubt the practical implications that such a vision of the future would bring to the field of regenerative medicine [29]. The generation of iPS cells from adult somatic cells currently remains a stochastically driven process of very low efficiency: of the lines produced, the majority harbour abnormalities, either karyotypic, epigenetic or in the form of somatic coding mutations [30]. These may render iPS cells tumorigenic and preclude their downstream clinical use [31, 32], emphasising the need to subject all new lines to rigorous screening and quality control. Such requirements inevitably make the process highly labour intensive while creating unrealistic timelines for the treatment of most disease states, even those that follow a more chronic aetiology. Furthermore, the investment of resources, both reagents and personnel, is significant, carrying with it an economic burden that few could sustain. Without a viable business model to follow, it is, therefore, unclear how iPS cell lines could ever be generated from patients on the kind of scale required to make any impact on the rising incidence of chronic and degenerative disease.

These pragmatic issues were recently brought into sharp focus by the regretable withdrawal of Geron Corporation from its stem cell programme, despite having been pioneers in the translation of regenerative medicine to the clinic. Furthermore, the company suspended its first-in-man trials using oligodendrocyte progenitors differentiated from hES cells for the treatment of spinal cord injury, for which economic, rather than scientific issues were cited as critical to the decision-making process. Significantly, the economic drivers were considered unfavourable, even though the programme made use of a single hES cell line that was fully characterised, and, more importantly, had already been granted regulatory approval for use in man. The added complexities of first generating and validating iPS cell lines for each patient to be treated, together with current uncertainty as to whether the products of differentiation, as well as the parent iPS cell line, would need to be subject to regulatory approval, is likely to render such a scenario untenable, at least for the foreseeable future. Indeed, it is the economics of

personalised medicines that have led many commentators to suggest that a more pragmatic approach to addressing the healthcare needs of the twenty-first century would be the construction of an extensive bank of fully approved and cGMP-compliant iPS cell lines [33], to which reprogramming technology is particularly well suited.

5.3.2 Construction of an iPS Cell Bank

A strong case in favour of using allogeneic iPS cells from an appropriate bank for the purpose of CRT, comes not only from the versatility of reprogramming protocols, but from cumulative evidence from several decades of research into whole organ transplantation, that has revealed the extent of the benefits that may be derived from the use of tissue typing to match patients with appropriate donors. In particular, it has become clear that the degree of matching required varies substantially between different tissues: whereas hematopoietic stem cell transplantation requires close identity between donor and recipient [34], significant leniency exists in the level of matching required for the successful transplantation of other tissues between individuals, such as the liver [35]. These empirical findings suggest that a continuum exists due to significant variation in the immunogenicity of tissues and their capacity for immune privilege: for instance, as the initial destination of foreign food-derived proteins transported from the gut, the liver may provide a natural microenvironment conducive to tolerance that actively suppresses inappropriate immune responses. Although empirical data are lacking to enable an objective assessment of where along such a continuum tissues differentiated from iPS cells might lie, the unique properties of the parent iPS cell line suggest that most of the tissues they spawn, whatever their identity, might be expected to exhibit relatively low immunogenicity.

First, by implementing protocols for the directed differentiation of iPS cells along defined lineage pathways, the resulting tissues are likely to be devoid of endogenous DC of donor origin [36], whose presence within whole organ allografts is known to initiate acute rejection via the direct pathway of alloantigen recognition. Furthermore, tissues differentiated from iPS cells, like those derived from hES cells [37], express barely detectable levels of MHC class I and class II determinants, depriving them of a source of alloantigens whose reprocessing and presentation via the indirect pathway would otherwise provoke a chronic form of allograft rejection. Most significantly, however, animal models of CRT have revealed that ES cell-derived tissues display a fragile yet demonstrable form of immune privilege that is shared by their iPS cell-derived counterparts, rendering them capable of securing acceptance across a single MHC class I disparity without recourse to any form of immune intervention [2, 38]. Although these properties are far from sufficient to prevent rejection across a fully allogeneic barrier [2], they support the notion that significant benefit may be derived from partial matching at the critical HLA loci, HLA-A, -B and -DR, as has been demonstrated for other

tissues that lie towards the lower end of the spectrum of immunogenicity, making the construction of a viable iPS cell bank a realistic objective, that avoids some of the difficulties encountered in the case of hES cells.

The production of a bank of hES cells is wholly dependent on the availability of blastocysts surplus to requirements in IVF clinics, and is, therefore, restricted by the genotype of the parents donating the embryo: while it is relatively easy to obtain cell lines for more common HLA haplotypes, the number of lines that would need to be derived to ensure representation from rarer haplotypes rapidly becomes untenable, falling foul of the law of diminishing returns. Furthermore, it is only after investing the time and resources into derivation of a novel hES cell line that its full MHC haplotype may be determined and its utility within the bank assessed accordingly. By contrast, iPS cell technology may be employed proactively to permit individuals with a desirable tissue type to be recruited as donors [39], recent advances in reprogramming technology lending themselves well to such an endeavour. Whereas, traditionally, iPS cell lines have been generated from small skin biopsies, causing only minimal discomfort to the donor, recent developments have demonstrated the feasibility of using non-mobilised peripheral blood [40], keratinocytes obtained from a single plucked hair [41] or even a small number of renal tubular epithelial cells isolated from urine [42], such non-invasive procedures raising few objections to the collection of the necessary samples.

By greatly simplifying the collection procedure, it may be possible to ensure the construction of a rather more comprehensive bank of iPS cell lines than of hES cells. In particular, it is likely to be possible to secure appropriate representation from all ethnic groups and minorities, rather than producing a bank strongly weighted towards the white Caucasian population that tends to frequent IVF clinics in Western societies. Perhaps most importantly, however, the ability to direct efforts towards specific individuals may make it feasible to recruit so-called ‘super donors’, homozygous at MHC loci, thought to be present within the population at a frequency of approximately 1.5 %. The availability of lines derived from such individuals, known to express a limited diversity of MHC determinants, would render them compatible with a far greater cohort of potential patients (Fig. 5.2). For example, working on the assumption that identity may only be required at two or more of the HLA-A, -B and -DR loci for a beneficial match to be identified, Taylor and colleagues estimated that as few as 150 hES cell lines might be required to make CRT available to a substantial proportion of the population. Importantly, this figure could be reduced to as few as ten hES cell lines if they had, by chance, been isolated from blastocysts homozygous at the MHC [43]. Although these calculations were based on simulated scenarios, Lin and colleagues have recently shown that among an established hES cell bank of 188 lines in China, eight were found to be homozygous at the MHC (4.2 %), yet contributed between 50 and 80 % of the total matching rates for the Huan population, which the IVF clinic had served [44]. Although such figures augur well for the generation of a bank of iPS cells capable of treating a significant proportion of the population, they are inevitably based on achieving only very limited identity between donor and recipient: given that a wide range of tissues might be differentiated from iPS

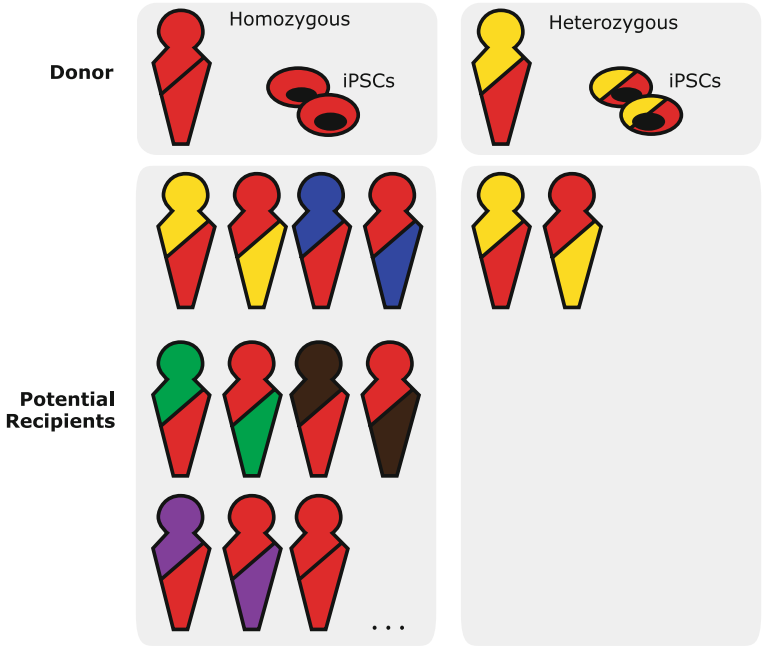

Fig. 5.2 iPS cell lines derived from 'super donors', homozygous at MHC loci, provide a valuable resource for CRT, since the cohort of potential recipients is significantly greater than for iPS cells from heterozygous donors

cells with varying immunogenicity, it remains doubtful that they will be accepted spontaneously without some form of additional immune intervention, of which immune suppression is undoubtedly the most commonly favoured.

5.3.3 Limitations of Immune Suppression in the Context of CRT

It would be difficult to underestimate the role played by immune suppression in enabling transplantation to become the treatment of choice for end-stage organ failure and it is for that reason that it is tempting to assume that the same immunosuppressive regimes may be applied within the context of CRT. Indeed, in a recent proof of principle study, the rejection of hES cells injected intramuscularly could be fully mitigated in a xenogeneic mouse model by the judicious use of a combination of sirolimus and tacrolimus [3]. Nevertheless, the uniqueness of the circumstances surrounding CRT suggests the need to carefully weigh the risks involved, which, paradoxically, may significantly exceed those of the very disease states amenable to treatment with stem cell-derived tissues, making a reliance on immune suppression ethically difficult to justify. While few would doubt that the

risks of long-term immune suppression are fully warranted in the case of organ failure, its use in CRT to facilitate the treatment of diseases such as age-related macular degeneration or Parkinson's disease, for which interventions to slow disease progression or alleviate symptoms are already available, results in a cost–benefit analysis that is rather precariously balanced.

First, many of the patients who might benefit from CRT in the future are likely to be elderly and especially vulnerable, therefore, to infectious diseases such as seasonal influenza, for which annual vaccination is strongly indicated. Long-term immune suppression would not only undermine the efficacy of such vaccination programmes but would confer on individuals significantly heightened susceptible to infection. Second, it is among the elderly that the incidence of malignancy is also greatest, further questioning the expediency of inhibiting the ongoing process of immune surveillance that subjects tissues to continual perusal, in order to seek out and destroy transformed cells. Importantly, the risks of malignancy are substantial for the recipients of tissues differentiated from pluripotent stem cells, due to the potential for carryover of residual undifferentiated cells within the inoculum that show a propensity for tumorigenesis [31, 32]. The fact that such legitimate concerns greatly restrict options available for mitigating the rejection of iPS cell-derived tissues has fuelled speculation that the autologous route to cell therapies may ultimately prove the only viable solution. Indeed, proponents of such a strategy point to recent technological advances that may help automate the process of iPS cell derivation, such as the increased efficiency of reprogramming and capacity for scale-up reported for the use of suspension cultures [45]. Such advances, it is argued, may ultimately reduce the costs and timelines involved to a level compatible with the use of iPS cells in a personalised manner.

5.4 The Case for Autologous iPS Cells

5.4.1 Reprogramming Induces the Ectopic Expression of Developmental Antigens

It is still rather too soon to predict whether greater automation of iPS cell derivation might streamline the process sufficiently to make reliance on autologous cell lines a realistic prospect. Irrespective of such optimisation, however, preference for the autologous route is wholly predicated on the assumption that self-tolerance would preclude the immunological recognition of autologous iPS cells and their derivatives, an assumption that has recently been challenged in a seminal study in mice.

Zhao and colleagues have shown that, whereas ES cells of B6 origin consistently formed teratomas when administered subcutaneously to syngeneic B6 mice, iPS cells generated from embryonic fibroblasts of the B6 strain, consistently failed to form viable teratomas in the same recipients [46]. In those cases in which

tetratoma tissue was rescued, there was evidence of significant necrosis, secondary to infiltration with $CD4^+$ and $CD8^+$ T cells. A comparison of the gene expression profile of teratomas derived from either cell type, when permitted to develop in immune compromised mice, revealed the ectopic up-regulation of several genes solely in iPS cell-derived tissues, including *Hormad1*, *Zg16* and *Cyp3a11*. The fact that the products of these genes served as the targets of the immune response, thereby conferring immunogenicity on otherwise autologous tissues, was elegantly demonstrated by genetically modifying B6 ES cells to express one or more of the putative antigens and, in doing so, rendering them vulnerable to the same immunological destruction as their iPS cell-derived counterparts. Furthermore, the authors directly demonstrated the presence of primed T cells capable of secreting interferon-γ when challenged with DC endogenously expressing Hormad1 [46].

Although little is known of the function of these genes, they each appear to be expressed very early during ontogeny, being down-regulated prior to development of the immune system: without their representation among the normal repertoire of self-antigens in utero, self-tolerance would not, therefore, have been fully established. If reprogramming to pluripotency were to erroneously up-regulate these developmental antigens whose expression persisted upon differentiation, the resulting tissues would harbour antigens that would inevitably be perceived as foreign. That Hormad1 may serve as a tumour-associated antigen due to its erroneous up-regulation in certain tumours, provides strong evidence in support of such a scenario [47].

Although the study by Zhao and colleagues has introduced an unexpected level of complexity to the debate surrounding the use of autologous iPS cell lines, these findings have yet to be independently verified. Furthermore, they have raised a number of important questions that still need to be addressed. Most importantly, the authors made use of embryonic fibroblasts for the derivation of iPS cells, which may have a rather greater propensity for expression of early developmental antigens than adult somatic cells [48]. Had adult fibroblasts been used instead, in order to more closely mimic the clinical scenario, the outcome may have been quite different. Second, the administration of undifferentiated iPS cells rather than purified cell types differentiated from them is likewise a significant departure from future clinical practise [49]. Most importantly, however, the question remains as to whether similar expression of developmental antigens might occur in response to reprogramming of human cells and, if so, whether they might also be recognised as foreign by the recipient immune system. Although evidence is necessarily circumstantial, Oct4-specific T cells have been reported within the memory T cell compartment of healthy individuals [50, 51], while patients suffering pre-neoplastic gammopathy have been shown to mount responses to Sox2 [52]. Importantly, Oct4 and Sox2 are two of the transcription factors required for efficient reprogramming to pluripotency, whose expression would normally be curtailed beyond the earliest stages of ontogeny: their recognition by the immune system under certain circumstances suggests, therefore, that similar issues of immunogenicity might be encountered were autologous iPS cell-derived tissues ever to be administered in man.

5.4.2 Direct Reprogramming: A Solution to Immunogenicity?

Given that the immunogenicity associated with autologous iPS cells appears to be an unanticipated consequence of recapitulating in adult somatic cells an early developmental state and its associated gene expression profile, the question arises as to whether avoiding a pluripotent state altogether might circumvent such issues. Interestingly, recent years have witnessed the publication of a number of elegant studies reporting the direct conversion of fibroblasts to therapeutically relevant cell types by the forced expression of key transcription factors [53]. For example, Wernig and colleagues showed the feasibility of generating functional neurons from mouse embryonic and postnatal fibroblasts by expression of the transcription factors *Ascl*1, *Brn*2 and *Myt1l* [54], while incorporation of a distinct cocktail of transcription factors—*Mash*1, *Nurr*1 and *Lmx*1*a*—consistently yielded dopaminergic neurons from both mouse and human fibroblasts [55]. More recently, a similar approach has been applied to the generation of hematopoietic cells [56] and even fully functional cardiomyocytes [57], a strategy that has even proven successful in vivo for the reprogramming of cardiac fibroblasts within their local microenvironment upon expression of *Gata*4, *Mef*2*c* and *Tbx*5 [58]. Importantly, the differentiation of each desired cell type was achieved without the need to navigate a pluripotent stem cell stage. While this omission may significantly impede efforts to scale up the ex vivo production of autologous cell types, which is heavily reliant on the self-renewing properties of iPS cells, it is interesting to speculate that the approach may avoid the up-regulation of developmental antigens responsible for rendering iPS cells and their progeny immunogenic, a prediction that will doubtless become the focus of future research endeavours. Even if such constraints could be circumvented, however, the treatment of many disease states would necessitate the genetic modification of autologous iPS cells in order to correct the gene defect driving the pathology, a strategy which inevitably risks creating further neoantigens to which an immune response may be directed.

5.4.3 Gene Correction and the Introduction of Neoantigens

There is little doubt that techniques for the genetic modification of human iPS cells to permit the correction of a specific gene defect are becoming well optimised, as evidenced by the recent use of human artificial chromosomes (HAC) to introduce a complete genomic dystrophin sequence into iPS cells derived from a patient with Duchenne muscular dystrophy, thereby restoring normal expression of the gene [59]. While such advances are clearly to be welcomed, it is important, nevertheless, to assess the likely impact on immunogenicity of cells and tissues differentiated from such sources (Fig. 5.3). Undoubtedly, the greatest risk comes from the restored expression of an entire missing gene, a scenario epitomised by some of the lysosomal storage diseases (LSD), in which the absence of specific lysosomal

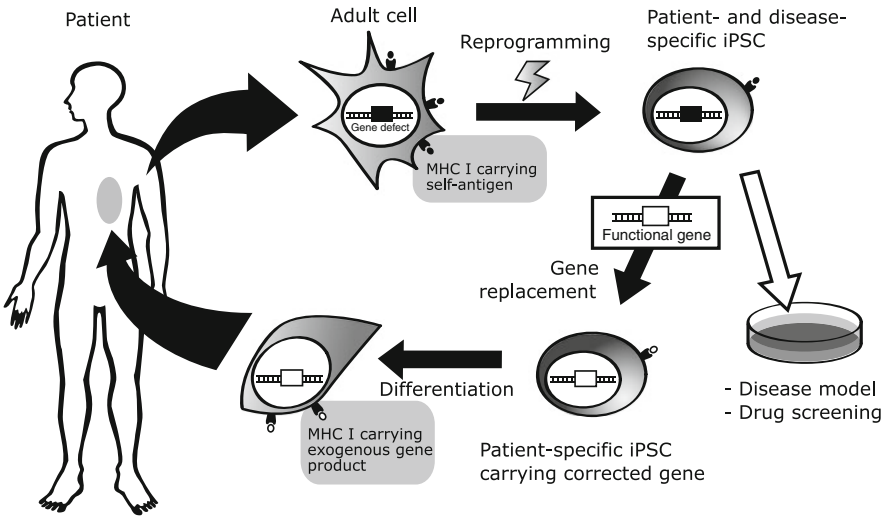

Fig. 5.3 Correction of a gene defect in patient-specific iPS cells risks the introduction of novel MHC class I-restricted epitopes to which the donor has not previously been exposed and is not, therefore, tolerant. Such genetic modification may, therefore, render administered cells immunogenic and vulnerable to immune recognition

enzymes results in the toxic accumulation of waste products with associated pathology. The implantation of autologous cells endogenously expressing the missing enzyme provides an attractive alternative to conventional enzyme replacement therapy, avoiding the need for repeated administration of the enzyme. Indeed, iPS cells have recently been derived from various mouse models of LSD [60] and from a patient with Pompe disease as a first step towards such a goal [61]. Nevertheless, the constitutive secretion of a 'foreign' antigen under such circumstances is likely to provoke the same neutralising antibody response that ultimately limits the efficacy of enzyme replacement therapy by actively inhibiting enzyme uptake by the mannose 6-phosphate receptor [62–64].

Other disease states may require the correction of a single mutation within a candidate gene; however, even such subtle modifications may inadvertently create novel epitopes capable of being presented to the T cell repertoire of the host in a conventional MHC-restricted manner. Indeed, naturally occurring polymorphisms in a wide variety of genes constitute an important source of so-called minor histocompatibility (mH) antigens whose cumulative effect is sufficient to induce rejection of tissues differentiated from mouse ES cells, even in recipients fully syngeneic at the MHC [2]. In mice of the H-2^k haplotype, the male-specific mH antigen, Dby, differs from its homologue on the X chromosome by only a few amino acids, two of which (positions 490 and 491), create an epitope that can bind to the MHC class II molecule, H-$2E^k$, and hence contribute to the rejection of male tissues by female recipients [65, 66]. These findings suggest that even the correction of point mutations in disease-related genes has the capacity to enhance the immunogenicity of iPS cell-derived tissues, although in a somewhat unpredictable

manner determined by the propensity for the novel epitopes to be liberated during antigen processing and their subsequent presentation by the MHC determinants of the individual in question. These variables necessarily introduce an unwelcome degree of uncertainty into the process of CRT.

5.5 Mitigating Immunogenicity: Prospects for Immune Intervention

There is little doubt that the spectre of immunological rejection has overshadowed the field of regenerative medicine since its inception and threatens to limit the utility of CRT in the future. Whereas the advent of induced pluripotency seemed to offer a solution to the issue of immunogenicity, it is now clear that such hopes were, at best, premature. While it is of little surprise that the use of allogeneic iPS cells would require some form of immune intervention were they ever to be used in a clinical setting, the unanticipated immunogenicity of cell lines derived in a fully autologous manner, has necessarily added a further level of complexity to the field. Despite these limitations, however, the immunological barriers involved are, for various reasons, less onerous than has previously faced the use of hES cell-derived tissues.

First, since the use of allogeneic iPS cells would help address some of the pragmatic issues surrounding the implementation of CRT, it is worth reiterating how the opportunity for generating a comprehensive bank of cGMP-compliant lines is far greater than would be achievable with hES cells. While targeted recruitment of donors, particularly those homozygous at MHC loci, remains a logical strategy, it might also be possible to exploit existing resources for such a purpose. There are, for example, an estimated 400,000 units of cord blood available worldwide that are fully immunologically characterised [67]: given the recent demonstration that iPS cell lines may be generated from umbilical cord blood using as few as two reprogramming factors (Oct4 and Sox2) [68], even after cryopreservation for up to 23 years [69], it is not beyond the realms of possibility that a bank of iPS cells could be produced in the future from such a resource that would permit a far greater level of matching than could ever be achieved with their hES cell counterparts.

Second, while autologous iPS cell-derived tissues have been shown to be surprisingly immunogenic, the nature of the immune response they elicit is quite distinct from conventional allograft rejection, driven by the recognition of polymorphic MHC determinants. Indeed, by being dependent on the erroneous upregulation of a few specific developmental antigens during the process of reprogramming, the response bears far greater similarity to autoimmunity than it does to allograft rejection. Significantly, the re-expression of developmental antigens in adulthood has long been hypothesised to be capable of initiating autoimmune responses and may, for instance, contribute to the aetiology of conditions such as myasthenia gravis. While our experiences over the past few decades reveal that it is not necessarily any easier to intervene in ongoing autoimmune disease than it is to inhibit transplant rejection, the peculiarities of CRT augur well for facilitating

immune intervention. In particular, the treatment of conventional autoimmunity requires the taming of a primed immune system, since by the time most patients present with symptoms, the response has already become fully established and subject to determinant spreading, while significant damage may also have been inflicted on the target tissue, causing associated loss of function. In contrast, by defining the timing of implantation of iPS cell-derived tissues, it is possible to determine when exposure of the host immune system to developmental antigens will first occur, providing an important window of opportunity for pre-emptive intervention, including the induction of antigen-specific tolerance. Such an approach would naturally constitute an attractive alternative to the use of immune suppression with its attendant risks and side effects. Nevertheless, given that the induction of immunological tolerance remains the holy grail of whole organ transplantation but has yet to impact the clinic, it is perhaps legitimate to ask whether there is any reason to believe that it may prove easier to achieve in the context of CRT than has been the case for organ transplantation.

Our own studies have demonstrated that mouse ES cell-derived tissues display an inherent capacity for immune privilege which permits the acceptance of tissues across an mH barrier without recourse to any form of immune intervention [2]. Furthermore, this form of immune privilege is capable of accommodating tissues that differ from the recipient at a single MHC class I locus, although the combination of class I and mH differences invariably precipitates rejection [38]. Interestingly, it is the constitutive expression of TGF-β_2 by ES cell-derived tissues that contributes to their immune privileged status by polarising infiltrating $CD4^+$ T cells towards a regulatory (Treg) phenotype [2], suggesting that protocols for tolerance induction that likewise favour Treg induction may synergise with the tissue's own capacity to repel deleterious immune responses. In support of such a contention, treatment of mice with a short course of non-depleting CD4 and CD8 monoclonal antibodies, known to facilitate the polarisation of Treg cells [70], permitted indefinite survival of tissues derived from fully allogeneic ES cells [2], even though any other tissue from the same donor would have been rejected, irrespective of such a conditioning regime. While a rigorous assessment of the immune privileged status of iPS cell-derived tissues has yet to be reported, our preliminary data suggest that they display similar properties to their ES cell-derived counterparts. Consequently, tolerance induction may prove a viable prospect for achieving the long-term engraftment of autologous iPS cell-derived tissues ectopically expressing developmental antigens, or even closely matched allogeneic lines selected from an extensive bank of iPS cells.

5.6 Conclusion

Although initial hopes that induced pluripotency had successfully circumvented the immunological barriers to regenerative medicine have ultimately proven unfounded, there remain significant advantages to the use of iPS cells in such a

context. Whether generated in an autologous manner or selected from a rather more extensive bank of cGMP-compliant lines than could ever be produced for ES cells, the degree of immunogenicity is likely to be restricted. Furthermore, the fact that iPS cells appear to contribute to their own acceptance through their inherent capacity for immune privilege, suggests realistic prospects for intervening in the rejection process through the induction of antigen-specific tolerance, thereby avoiding any reliance on alternative forms of immune intervention, such as the use of immune suppression. Although the immune system of the recipient is, therefore, a formidable opponent in the context of CRT and should never be underestimated, there is, nevertheless, hope that induced pluripotency may yet help to resolve this enduring barrier and unlock the benefits of regenerative medicine in the future.

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Chapter 6
Thymic Involution: A Barrier or Opportunity for Cell Replacement Therapy?

Simon Hackett and Paul J. Fairchild

Abstract Although regenerative medicine offers prospects for cell replacement therapies relevant to many disease states, research has mainly focused on regenerating major organ systems such as the heart, kidney and lungs. The thymus, however, undergoes natural age-related involution and its ability to sustain a functional T cell repertoire therefore declines throughout life. While in healthy adults this process has no significant immunological impact, in immunocompromised patients, the involuted thymus is unable to rescue immune homeostasis which leads to increased risk of infection. Regenerating the thymus using stem cell technology, may, therefore, provide a viable option for rescuing immune function in immune compromised or elderly patients. Furthermore, thymic regeneration offers the prospect of influencing the acceptance of allogeneic tissues through the induction of central tolerance. Here, we explore the rationale behind thymic transplantation and current efforts in the stem cell field aiming to derive functional thymic tissue.

6.1 Introduction

The ability of pluripotent stem cells to differentiate into any cell type in the human body holds promise for their use in treating diseases for which current pharmacological methods have failed. However, contrary to the often misleading media coverage regarding stem cells, there are various hurdles that must be overcome

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before these cells will have any major clinical impact. Without doubt, the most significant outstanding issue is their likely rejection by the host immune system.

The immune system is one of the fundamental systems in organisms required for homeostatic regulation and, in particular, defence against pathogens. It is widely accepted that ageing is associated with a decline in immune responsiveness, to which involution of the thymus constitutes a significant contributing factor. The thymus undergoes age-related changes throughout life which are accelerated at the onset of puberty, due to the release of sex hormones. By the age of 70, the thymus weighs less than 5 g and has correspondingly poor function [1], indeed, the reduced immune response from elderly populations following vaccination is directly linked with thymus involution. This arises from a decline in T cell production leading to an increased risk of opportunistic infections such as *Pneumocystis carinii* and also an increased risk of cancers stemming from the inability of T cells to recognise tumour-associated antigens. The problems presented by thymic involution have led to various attempts at rejuvenating thymic function using a variety of approaches, one of which involves the use of stem cell-derived tissues.

There has been much interest in differentiating from pluripotent stem cells, a range of tissue types including cardiomyocytes, skeletal muscle and hepatocytes. It has been hypothesised that thymus replacement therapy, using stem cell technology to produce thymic epithelial progenitor cells (TEPC), could revolutionise treatments, not only for age-related atrophy of the thymus, but for a host of other athymic-conditions, such as DiGeorge syndrome. Additionally, thymus transplantation could aid in recovering T cell function in immunocompromised patients as a result of chemotherapy or the pathogenesis of AIDS. Furthermore, given that the thymus is responsible for the establishment of central tolerance, a thymus generated from donor stem cells may also induce tolerance to alternative tissues derived from the same parental stem cell source. This chapter will explore the feasibility and potential impact of thymic regeneration. However, in order to elucidate a coherent strategy for thymic regeneration, it is important to first understand its development and function.

6.2 Thymic Development: A Historical Perspective

The thymus was first described by Galen of Pergamum in Ancient Greece around 150 AD [2]. However, its true function was elucidated only 50 years ago when Jacques Miller showed that removal of the thymus in mice, resulted in a deficiency of lymphocyte production [3]. The thymus is located in the upper anterior area of the thorax and consists of two distinct anatomical regions: the medulla and the cortex. The function of the thymus is to sustain the maturation of T-lymphocytes, produced as early progenitors in the bone marrow, which play a central role in the adaptive immune system, preventing infection from pathogenic microorganisms. T cell precursors, produced by the bone marrow, undergo differentiation upon

entering the thymus, eventually producing mature T cells through the interactions with several components of the thymus and, in particular, the thymic epithelial cells (TECs). During this process, the medulla and cortex serve as the sites of positive and negative selection of T cells, respectively. The fate of a T cell depends upon the specificity of its cell surface antigen receptors. T cells which express a T cell receptor (TCR) with little affinity for products of the major histocompatibility complex (MHC), are neglected and are, therefore, lost from the developing repertoire. In contrast, T cells expressing a TCR with high affinity for self-MHC molecules undergo negative selection and are, therefore, actively eliminated. Rare T cells which survive these complementary processes are induced to adopt a mature $CD4^+$ or $CD8^+$ phenotype, prior to their emigration from the thymus to populate the periphery [4].

Involution of the thymus begins at 6–12 months of age and continues throughout life, although it is greatly accelerated at puberty [5]. Around the age of 60, *bona fide* thymus tissue becomes largely indistinguishable from surrounding adipose tissue in the thoracic cavity. The maturation of T cells declines steadily from puberty onwards, during which the production of sex hormones, in particular gonadotrophin, have long been linked with thymic involution [6]. The process of involution itself is mediated by various factors, including the expression of *Fas* by TEC, the ligation of which leads to programmed cell death by apoptosis [7]. The exact molecular mechanisms of thymic involution are not fully understood, however, key research by Sutherland et al. [8] showed that inhibition of the sex hormone androgen results in regeneration of the adult mouse thymus and restoration of peripheral T cell phenotype and function. This has been considered a possible treatment for involution and studies have shown that elderly patients treated for prostatic cancer by sex steroid ablation demonstrate an increase in peripheral T cell counts, as evidenced by identification of TCR excision circles (TRECs) among circulating peripheral T cells.

Thymic function can also be further damaged by cytotoxic drugs, particularly immunosuppressive agents such as cyclosporine A. Sakaguchi and Sakaguchi [9] showed that transplantation of cyclosporine A treated thymi into *nude* mice caused organ-specific autoimmunity. The authors concluded that cyclosporine A interferes with the thymic production of certain regulatory T cells which control self-reactivity. These self-reactive T cells subsequently expand, inducing autoimmune disease. As mentioned earlier, thymic regeneration in patients treated with such immunosuppressive drugs may be beneficial in restoring thymic function.

6.3 Thymic Involution: The Reasoning Behind the Process

At first sight, thymic involution may appear an unlikely evolutionary adaptation. However, various theories have been expounded to explain why evolution may have encouraged the reduced thymic function seen with advancing age. It has, for instance, been suggested that involution could have arisen as a direct adaptation to

reduce the likelihood of malignancy. The thymus initially sustains a rate of cellular proliferation not witnessed in any other organ during the neonatal period. Furthermore, given that T cell development is also associated with complex rearrangements of germline genes, there is a high probability of transformation. Consequently, decreased thymic output and replacement of the thymic architecture with adipose tissue could, therefore, represent a mechanism to reduce cellular proliferation and prevent the formation of thymomas [10]. However, there is no evidence to show that removal of the thymus from adult or neonatal mice leads to any advantageous outcome: indeed, such procedures increase the risk of autoimmune conditions and, in the absence of a functional T cell compartment, the incidence of malignancy increases rather than declining, thereby undermining any selective pressure.

The other major contesting theory which aims to explain thymus involution has been dubbed the 'disposable soma' theory. Kirkwood [11] argues that, from an evolutionary perspective, the most energy-efficient organisms are most likely to survive. Organisms must evolve to develop a balance of energy for vital and non-vital functions, thereby conserving energy which will, in turn, maximise their capability of producing viable offspring. As discussed by George and Ritter [10], the organism is therefore likely to expend most energy on maintaining the germline, which is vital for future viability of the species. Whether or not the conservation of energy resulting from thymic involution is advantageous is, however, a matter of some debate. Organisms constantly need to adapt to new environments with the possibility of exposure to new pathogens. From this point of view, the involution of the thymus is a disadvantage as a more restricted diversity of the T cell repertoire occurs with age, as homeostatic proliferation of T cells sustains T cell numbers in the periphery, replacing the de novo export of T cells from the thymus. Although the exact reasons for thymic involution will doubtless remain a matter of conjecture, its impact on the ageing immune system is beyond doubt and remains a significant unmet medical need. The remainder of this chapter will, therefore, discuss possible approaches to rejuvenating the involuted thymus that emanate from a detailed understanding of thymus development.

6.4 Thymus Development

6.4.1 Embryogenesis of the Thymus

The evolutionary appearance of the thymus is linked phylogenetically with acquisition of the jaw, perhaps suggesting that its advent may be due to the dietary changes associated with jaw development, as well as the associated increase in injury and infection. This would, in turn, create a selective pressure to develop an organ with a high immunological potential which could produce diverse immune cells to aid in the targeting and destruction of pathogens. The diversity produced

by recombination of V, D and J region gene segments, along with the subsequent selection of a functional T cell repertoire, allows development of the self/non-self discrimination that is central to the adaptive immune system. The thymus is fundamental to this function, with the cortex and the medulla responsible for positive and negative selection of mature T cells respectively.

Ontogenetically, the thymus originates from the pharyngeal pouches that derive from the endodermal lineage. In sharks, the thymus originates from a variety of pharyngeal pouches whereas in the chicken, it forms from an outgrowth of the third and fourth pharyngeal pouches. In mice the third pharyngeal pouches form a primordial structure which is surrounded by a capsule of mesenchymal origin which will further develop into the thymus proper and the closely associated parathyroid gland [12].

The embryological development of the thymus may be divided into a number of discrete events [13]: (1) positioning, (2) budding and outgrowth of the thymus from the relevant pharyngeal pouch, (3) detachment of the primitive thymus from the endoderm and (4) patterning, differentiation and migration of the thymus to its anatomical location in the thoracic cavity. Thymus development is strongly linked with the expression of the transcription factor FoxN1. FoxN1 is initially expressed at day E11.25 of gestation. Mutations and knockout of the gene result in athymia with arrest of the thymic primordium at day E11.5–12.5 [13, hematopoietic 14]. FoxN1 expression has also been shown to be essential for colonisation of the developing thymus with elements [13]. Genes such as Hoxa3, Pax3 as well as FoxN1 correlate with athymic or thymic hypoplasia phenotypes, as discussed below [15].

6.4.2 Developmental Origin of TECs: Endoderm or Ectoderm Derivation?

The embryological germ layer of origin of TECs has been widely disputed, as has the relationship between cortical and medullary TECs (mTECs). One possible model, described as the dual-origin model, suggests that the thymus derives from both the endoderm and ectoderm, the cortical TECs (cTECs) deriving from the ectoderm and the mTECs showing allegiance to the endoderm lineage [16]. Early studies by Cordier and Heremans drew two pertinent conclusions: the thymic deficiency observed in *nude* mice arose due to the failure of the ectodermal cells to produce a cervical vesicle and thereby contribute to thymic formation. Second, the endodermally derived component of the thymus fails to develop due to the resulting deficiency in signals of ectodermal origin. This initially suggested that contributions from both the endoderm and ectoderm were necessary for thymus generation.

The second model arises from seminal work carried out by Le Douarin and Jotereau [17], which suggested that the thymus arises from the endoderm alone. In

a series of elegant experiments, the authors used chick-quail chimeras, differences in the structure of the interphase nuclei allowing them to distinguish between cells derived from the two species. The pharyngeal endoderm was isolated from quail embryos before the development of the third pharyngeal pouch and transplanted into chick embryos. The results corroborated previous research from Moore and Owen [18] demonstrating an extrinsic origin of lymphoid stem cells in the thymus. Additionally, it showed that purified pharyngeal endoderm is able to generate a thymus with the distinct structural compartments of a medulla and cortex. This therefore added substantial weight to the hypothesis that the thymus arises solely from the endoderm, a conclusion which was further supported by more recent work by Gordon et al. [19] who showed, through histological analysis, that, although the third pharyngeal pouch endoderm and ectoderm make contact around embryonic day E10.5–11.5, these tissues subsequently separate, accompanied by apoptosis of cells in the connecting region.

6.4.3 Do Cortical and Medullary Epithelial Cells Share a Common Progenitor?

In the light of compelling evidence in support of a single germ layer of origin of the thymus, it might be predicted that cTEC and mTEC ultimately share a common progenitor, a prediction that has since been verified by two landmark papers, one by Bleul et al. [20] and the other by Gordon et al. [21]. These authors used cell lineage-tracing experiments, based on the Cre/loxP system, to determine the fate of genetically marked TECs. To determine Cre activity, Bleul et al. used a Rosa26R-eYFP reporter system that results in fluorescence after Cre-mediated chromosomal rearrangement. A total of 58 thymic lobes derived from 29 mice were sampled and 21 clusters identified containing $eYFP^{+}$ cells. The function of postnatal progenitor cells in the thymus was probed by reverting a mutant allele of *Foxn1* to its wild-type form in vivo. This resulted in the formation of small thymic lobes containing both medullary and cortical epithelial progenitor cells, thereby indicating that single epithelial progenitors can give rise to a functioning thymic microenvironment.

Since the pioneering work of Le Douarin [17], there has been extensive interest in the origin of TECs. Further evidence in favour of the common progenitor hypothesis for cortical and medullary epithelial cells was provided by Rossi et al. [22]. The authors used disaggregated E12 thymic rudiments from mice and identified epithelial cells using the marker pan-cytokeratin. They compared pan-cytokeratin labelled cells with expression of EpCAM1, a marker for medullary and cortical epithelial cells. The relative level of EpCAM1 expression is linked to the TEC types: high EpCAM1 expression is typical of mTECs, whilst low EpCAM1 expression correlates with a cortical phenotype. The authors found that all pan-cytokeratin positive cells from day E12 expressed comparable levels of EpCAM1 and had yet to differentiate into medullary or cortical epithelium. Therefore,

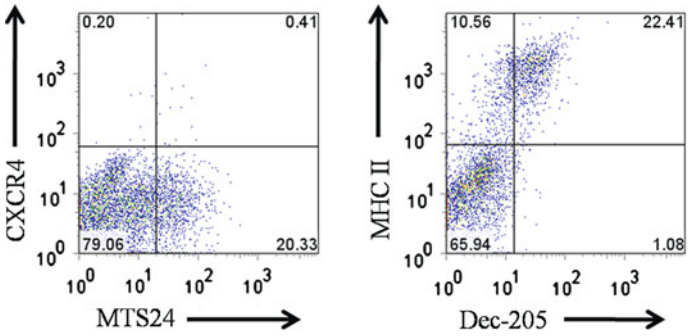

Fig. 6.1 Flow cytometric analysis of thymic epithelial cells (TECs) following collagenase dispersion of embryonic thymus from day E15 foetal mice. Surface staining for MHC class II and the cTEC restricted marker, Dec-205, show commitment to the cortical lineage at this stage of gestation, while staining for MTS24 reveals the presence of a discrete population of TECPs (Georgiadou and Fairchild, unpublished observations)

EpCAM1 can be used as an epithelial progenitor cell marker. Armed with this information, Rossi and colleagues injected single EpCAM1$^+$ progenitors from mice constitutively expressing yellow fluorescent protein (YFP) into early thymic rudiments from embryos at E12 of gestation and were grafted under the kidney capsule of syngeneic recipients. Subsequent immunohistochemical analysis of recovered thymi 4 weeks after grafting, revealed TECs of both cortical and medullary phenotype expressing YFP as evidence of their differentiation from the original donor cell. From these experiments, the authors were able to show unequivocally that medullary and cortical TECs derive from a common precursor in a clonogenic fashion, finally disproving the hypothesis that the thymic epithelial lineages arise from different germ layers during ontogeny.

In addition to EpCAM1, the marker MTS24 has been shown to be specific for TEPC in the early thymus (Fig. 6.1). It was shown that all pan-cytokeratin-positive cells were also MTS24$^+$ at day E12. Using this marker, Bennett et al. [23] demonstrated that purified populations of MTS20$^+$/MTS24$^+$ cells were able to differentiate into all known types of TECs, attract lymphoid progenitors and support development of CD4$^+$ and CD8$^+$ T cells in nude mice following ectopic grafting under the kidney capsule. This suggests that an entire functional thymus can be obtained from a discrete population of progenitor cells, which augurs well for regenerative medicine strategies.

6.4.4 T Cell Repertoire Selection

Given that T cells are involved in both humoral and cell-mediated immunity and have a powerful role in aiding the destruction of unwanted pathogens and tissues, their homeostatic regulation in the body is tightly controlled by the thymus

through the process of T cell repertoire selection. Thymocyte progenitors develop from hematopoietic stem cells in the bone marrow and, on reaching the thymus, are expanded through direct contact with TECs. Thymocytes are categorised according to their expression of CD4 and/or CD8. The earliest thymocytes are both CD4 and CD8 negative (termed $CD4^{-}CD8^{-}$ or "double negative" (DN) thymocytes). During this stage, the β-chain of the TCR undergoes rearrangement and the ensuing process of β selection encourages proliferation of thymocytes and culminates in α chain rearrangement, leading to coordinated upregulation of both CD4 and CD8 and acquisition of a "double positive" phenotype. Following rearrangement, thymocytes undergo positive and negative selection, which, unlike β selection, is not associated with proliferation.

Positive selection occurs predominantly in the cortical area in the thymus which expresses high levels of self peptides bound to MHC molecules at the surface of cTECs. A proportion of thymocytes binds to the complexes with low affinity which promotes their survival and subsequent homeostatic expansion in the periphery. Thymocytes which do not have any affinity for self peptides are eliminated from the T cell repertoire through apoptosis in a process sometimes referred to as "death by neglect". Following this process, surviving thymocytes undergo negative selection in the medulla which is highly populated by professional antigen presenting cells, including dendritic cells, which present a comprehensive array of self peptides, against which self-reactivity may be screened.

Negative selection is one of the body's key defences against autoimmunity and recent work has shown that the *Autoimmune Regulator (Aire)* gene plays an important role. The importance of *Aire* in the prevention of autoimmunity was first demonstrated by Anderson et al. [24] who showed that mutations in this transcription factor resulted in organ-specific autoimmune destruction, a condition known as Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy (APECED). Using *Aire*-deficient mice, the authors were able to recapitulate some aspects of the disease and, in particular, the autoimmune component. The condition can be explained by the role that *Aire* normally plays in negative selection of thymocytes since it acts to control the expression of tissue-specific antigens by mTECs. As such, *Aire* functions to increase the transcription of genes that are usually only expressed in peripheral tissues, a process known as promiscuous gene expression. T cells with receptors complementary to these peripheral antigens can, therefore, be purged from the T cell repertoire, thereby preventing the emigration of autoreactive T cells to the periphery. Importantly, in the *Aire*-deficient mice generated by Anderson et al. [24], the mTECs in the thymus lacked not only expression of *Aire* but also showed a reduction in the expression of genes encoding peripheral proteins, known to serve as key autoantigens.

Collectively, positive and negative selection allow only T cells with a functional TCR devoid of self-reactivity to colonise the periphery. It is estimated that less than 3 % of cells that succumb to repertoire selection in the thymus emerge as so-called "recent thymic emigrants" (RTEs). Although alternative mechanisms of tolerance exist in the periphery, the thymus is, nevertheless, the primary site of selection.

6.4.5 T Cell Receptor Excision Circles (TRECs): A Measure of Thymic Output

For many years, measuring thymic output of newly developed T cells was not possible due to the lack of a specific marker of RTEs. A crude way of measuring thymic output was, therefore, to assess thymic volume which correlates with thymic function, although rather imprecisely, due to progressive invasion of the thymus by adipocytes with increasing age. It was only when Douek et al. [25] showed the practical applications of TRECs that it became possible to directly measure thymic output. TRECs are episomal DNA circles that are produced during rearrangement of TCR chain genes, thereby bringing into close juxtaposition V, D and J region gene segments. TRECs have been shown to be stable, are not duplicated during mitosis and are, therefore, proportionally diluted from the T cell repertoire upon proliferation. Since TRECs can also be detected among T cells from older individuals [26], their properties make them a useful tool in measuring thymic output throughout life. Using this approach, Douek et al. [25] successfully showed that, although thymic function progressively declines, the thymus still maintains residual function and outputs into adulthood and old age. They were also able to show that HIV infection leads to a decrease in thymic function measured by TREC levels and that treatment with highly active anti-retroviral therapy (HAART) increased thymic output in HIV-infected patients, thereby demonstrating the usefulness of TRECs in measuring RTEs and, therefore, thymic function. Consequently, TRECs provide a useful means of determining whether a thymus which has been rejuvenated, has the capacity to support T cell selection and development.

6.5 Thymus-Based Strategies for Immune Intervention

6.5.1 Thymic Transplantation

Given that the thymus atrophies progressively with age and, in some genetic conditions such as DiGeorge syndrome, is absent altogether [27], the practicalities of thymus transplantation have been widely explored. Thymic transplantation in athymic patients would, at least theoretically, increase the patient's T cell population with a concomitant acquisition of immune competence. Perhaps the most notable attempt at thymic transplantation was carried out my Markert et al. [27] who treated five infants with complete DiGeorge syndrome by transplanting cultured postnatal thymus tissue. Following transplantation, T cell proliferative responses to mitogens developed in four out of five patients. Despite only two patients surviving (three having died from infection and/or abnormalities unrelated to transplantation) biopsies of thymus tissue showed normal histological characterisation, consistent with the presence of TECs and with active T cell production. In three of the trial patients, T cells could be detected around one month post-transplantation.

The authors published a follow-up study in 2003 [28] showing the progression of patients who had undergone thymus transplantation. Of the 12 patients studied, 7 lived at home 8.5 years following transplantation. Furthermore, all seven survivors showed T cell proliferative responses to mitogens, and B cell function developed in the three patients who were tested. The patients who did not survive died from causes related to functional abnormalities of DiGeorge Syndrome, such as heart defects.

From the evidence presented by Markert et al. [27] thymic tissue transplantation is a viable option for recovering T cell production in patients affected with DiGeorge syndrome. However, as thymus grafts were allogeneic in origin, the patients were treated with immunosuppressive drugs such as methotrexate. This results in dampening of the very immune system which thymus transplantation seeks to restore, which, over time, may prove detrimental. Furthermore, such a treatment regime is limited by a paucity of donor tissue as well as the risks associated with highly invasive surgery, the latter often proving dangerous in immunocompromised patients.

6.5.2 Mixed Chimerism and Tolerance

Chimerism is used to describe the state whereby an organism contains cells derived from two or more distinct individuals [29]. Patients undergoing treatment for hematological malignancies often undergo radio ablative and chemotherapeutic treatment in advance of bone marrow transplantation. In addition to wiping out these malignancies, treatment also eradicates normal hematopoiesis and lymphopoiesis. Destroying the recipient's own bone marrow, eliminates cells that could cause rejection of the donor bone marrow inoculum. Upon transplantation of donor bone marrow, the resulting hematopoiesis may be 100 % of donor origin (complete chimerism) or a mixture of both donor and host-derived cells (mixed chimerism). Given that progenitor cells migrate from the bone marrow and populate the thymus, where they develop into dendritic cells [30], mixed chimerism results in the deletion of host-reactive and donor-reactive T cells, thereby rendering the individual tolerant to both host and donor cells. Chimerism has been considered as a targeted method of inducing immunological tolerance to donor cells at source rather than simply a naturally occurring phenomenon associated with bone marrow transplantation.

6.5.3 Deriving Thymic Progenitor Cells from Stem Cells

As previously discussed, deriving a thymus from stem cells would be advantageous on two counts: first, it would replace an already deficient or missing thymus, and second, it might permit the induction of tolerance to alternative tissues differentiated

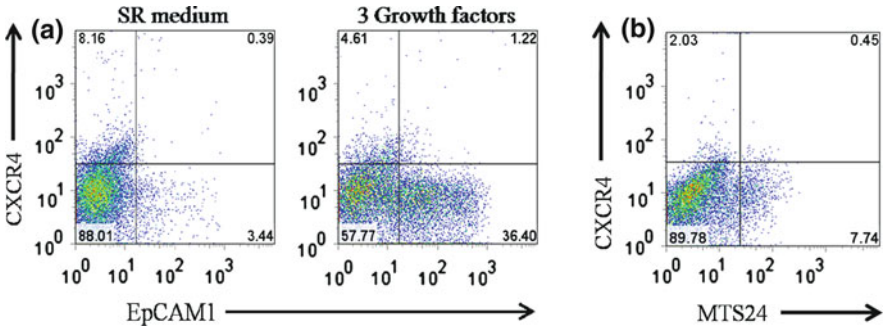

Fig. 6.2 Differentiation of putative TEPC from mouse ES cells. **a** ES cells were cultured for 7 days on tissue culture plastic previously coated with extracellular matrix (ECM) proteins secreted by a monolayer of mouse embryonic fibroblasts, which were subsequently removed by EDTA treatment. ES cells were permitted to differentiate spontaneously in either unsupplemented serum replacement (SR) medium or SR supplemented with 50 ng/ml of epidermal growth factor (EGF), 20 ng/ml of fibroblast growth factor (FGF)-7 and 20 ng/ml of bone morphogenetic protein (BMP)-4. The combination of growth factors was consistently shown to favour epithelial cell commitment, as determined by surface expression of EpCAM1. **b** When ES cells were permitted to form embryoid bodies for 6 days (the final 3.5 days in SR medium to exclude hematopoietic growth factors) before plating on ECM in the presence of EGF, FGF-7 and BMP-4, a discrete population of $MTS24^+$ progenitor cells was obtained (Georgiadou and Fairchild, unpublished observations)

from the same parental stem cells. As described above, there is now irrefutable evidence for the existence of a common TEPC for both medullary and cortical TECs, which has recently been derived from mouse ES cells [31]. However, the difficulties in deriving such a population of cells for therapeutic purposes should not be underestimated: TEPCs are a trace population which makes their isolation particularly demanding. In addition, it is often difficult to encourage these thymic progenitor cells to form a functioning thymus in vivo. Despite the technical difficulties of such a procedure, Lai et al. [31] were able to isolate populations of thymic progenitor cells in vitro from mouse ES cells using two markers: CXCR4 and MTS24. CXCR4 has been shown to be expressed by cells of the definitive endoderm [32], from which TECs are known to arise. Furthermore, given that MTS24 has been shown to be expressed at day E12 of gestation during development of the thymic epithelium [33] and has been shown to define a bipotent progenitor capable of generating both mTECs and cTECs, CXCR4 and MTS24 together are useful markers for identifying thymic progenitor cells among the differentiated progeny of ES cells (Fig. 6.2). Following isolation of thymic progenitor cells, Lai et al. transplanted the cells under the kidney capsule of recipient mice, where they formed a functioning thymus-like organ which sustained an increased peripheral $CD4^+$ T cell repertoire.

In addition to this work, Seach et al. [34] have shown that TECs derived from embryonic day 15 thymic grafts can also form a physiologically functioning thymus in mice. The authors used silicone chambers containing murine thymic grafts which were transplanted into the inguinal fat pad of athymic Balb/c *nude* mice. The authors showed that the thymic grafts became vascularised and remained viable. The grafts were also shown to be functional, as demonstrated by

increased peripheral $CD4^+$ and $CD8^+$ T cell counts, compared with controls. Furthermore, the functionality of the newly selected T cells was demonstrated by successful rejection of MHC-mismatched skin grafts. The authors clearly show that the microenvironment is important for the vascularisation and development of thymic tissue transplanted into a host. Approaches which tackle not only the immunological basis but also the practicalities of transplantation will aid in the successful development of treatments for thymic rejuvenation.

6.5.4 iPS Cells: A Need for Central Tolerance?

The technology of induced pluripotent stem cells (iPSC) was first demonstrated in 2006 by Takahashi and Yamanaka [35] and offers the opportunity to develop patient-specific stem cell therapies by deriving stem cells from the somatic cells not only of adults but also from elderly patients. This technology may bypass the need for the induction of central tolerance since autologous cells are less likely to be targeted by the immune response than ES cells and their progeny. Nevertheless, there are many questions which still surround iPS cells such as their stability and capacity to form tumours.

More recently, Inami et al. [36] have shown that it is possible to derive TECs from iPS cells. The cells were cultured for 4 days with several growth factors and differentiated along the endodermal lineage. The group then treated the cells with a cocktail of growth factors which are present during embryological development of the thymus, Fgf8, Fgf7, Fgf10 and BMP4. Treatment of iPS cells under these conditions, resulted in the formation of TEPCs, identified by expression of MTS24 and EpCAM1. The TEPCs isolated from these cultures differentiated into mTECs following treatment with lithium chloride and receptor activator nuclear factor B ligand (RANKL). These findings, like those of Lai and Jin [32], directly demonstrated the feasibility of deriving thymic progenitor cells from stem cell populations and differentiating them into TECs. Nevertheless, this study identified TECs solely on the basis of their phenotype and did not show that TECs obtained in this way were physiologically functional or could form a thymic graft in vivo. Nevertheless, this work suggests that, in the future, rejuvenation of the thymus in an autologous manner may prove feasible, thereby not only avoiding rejection, but ensuring the development of a truly self-MHC restricted T cell repertoire.

6.6 Concluding Remarks

Despite the fact that the molecular mechanisms behind thymic function have been extensively studied, there has been only minimal success in securing true thymic rejuvenation. Although attempts at sex steroid ablation and localised growth factor treatment have yielded some success, the increase in thymic output is short-lived.

Stem cell therapies offer the promise of providing lasting therapies for diseases with limited treatment options. Using stem cells to derive thymic tissue may sustain increased thymic output into old age. Current models of rejuvenation of the thymus in the mouse have yielded promising results suggesting that, in the future, similar techniques may be translated for use in human patients, opening up the possibility of novel methods for treating immune compromised patients as well as permitting the better control of transplantation tolerance.

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Part II
Strategies for Overcoming Rejection of Stem Cell Grafts

Chapter 7
Construction of Human Embryonic Stem Cell Banks: Prospects for Tissue Matching

Ge Lin, Qi Ou-Yang, Xiaobing Qian and Guangxiu Lu

Abstract Although human embryonic stem (hES) cells are critical for the future of regenerative medicine, their clinical application is threatened by polymorphism within the human leukocyte associated antigens (HLA), that normally precipitate rejection. Given that HLA matching between donor and recipient reduces the immune response in conventional transplantation, establishment of a hES cell bank with a broad spectrum of HLA genotypes may provide greater access to cell-replacement therapies. Both theoretical calculations and actual HLA matching analysis between an established hES cell bank and local populations indicate that a feasible number of hES cell lines could provide sufficient HLA matched tissues for the majority of the population. Furthermore, isolated hES cell lines with homozygous HLA haplotypes will significantly reduce the number of lines required, parthenogenic and "unwanted" clinical embryos serving as two major sources. We will discuss prospects for hES cell banking and issues involved in clinical compliance in the light of recent developments in induced pluripotency, using a patient's own somatic cells.

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7.1 Introduction

Since Thomson and colleagues first derived human embryonic stem (hES) cells from blastocysts in 1998 [1], they have been considered the best seed cells for replacement therapy to treat degenerative diseases in multiple organs. However, a major obstacle to eventually use hES cells in clinical applications is the immunological incompatibility between hES cell derivatives and potential recipients, which will lead to immune rejection of the transplants [2–4]. Several strategies have been suggested as a way of solving this problem [5], most of which will be discussed in detail in other chapters. Here, we will focus on the feasibility of banking sufficient hES cell lines immunologically matched to a population and address several important issues related to establishment of a clinical-grade hES cell bank.

7.2 Polymorphism of the Major Histocompatibility Complex and Need for Tissue Matching

Stem cell transplantation and therapies are conducted with two types of tissue sources. Autologous transplantation can be performed without risk of immunological rejection, for example, with hematopoietic stem cells [6, 7], mesenchymal stem cells (MSCs) [8–10], and autologous cartilage cells [10, 11]. In these cases, the donor is also the recipient and would obviously not reject the tissue. On the other hand, allogeneic stem cell therapies with umbilical cord blood [12], MSCs [13], skin substitute [14], or hES cell derivatives face the challenge of immunological rejection due to tissue type incompatibility.

The molecules which primarily mediate the immunological responses to foreign tissue are the human leukocyte associated antigens (HLA) encoded by the Major Histocompatibility Complex (MHC) genes located in the short arm of human chromosome 6 (6p21.3) [15, 16]. HLA genetic polymorphism is the major reason for immune rejection. The HLA class I (A, B, C) and HLA class II (DR, DP, DQ) genes contain the most polymorphic loci in the human genome and directly affect the outcome of cell, tissue, and solid organ transplantation [17, 18]. As of Jan 2010, there were 965 alleles found in the HLA-A locus, and 1,543, 626, 762, and 107 alleles in the HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1 loci, respectively [19]. The high rate of HLA polymorphism at these loci renders the probability of finding two HLA-identical individuals at all loci very low. Therefore, HLA matching is a common method for reducing the chance of immune rejection in clinical allogeneic organ or stem cell transplantation by searching for donors with the appropriate degree of HLA matches and combining the therapy with immune suppressive drugs.

Transplantation of different organs or tissues requires different levels of matching. For example, hematopoietic stem cell transplantation needs high

resolution HLA matching (equivalent to nucleotide-sequence level typing) [20], while low resolution HLA typing (conventional serological typing) is sufficient for kidney transplantation [21], and cornea transplants may not require HLA matching due to immunological privilege in the anterior chamber. Nevertheless, for most tissue and solid organ transplantation, HLA matching is an indispensable process in transplantation and could improve the transplant survival rate.

7.3 Stem Cell Banking for HLA Matching

Several methods have been used to identify HLA matched donors in allogeneic stem cell or solid organ transplantation. For sibling donor transplantation, there is a 1 in 4 chance that the sibling will be HLA-identical. For parental or filial donor transplantation, the recipient may be only HLA haploidentical; that is, having complete HLA matches on only one chromosome. In the absence of such a related donor, establishment of a cell bank or a database with rich HLA types, such as the umbilical cord blood bank [22, 23], bone marrow donor registry [24], or organ donation registry is critical. Stem cell banks have been used for transplantation for more than 20 years. Many bone marrow registry organizations were established in the late 1980s all lover the world, and the New York Blood Center established the first umbilical cord blood bank in 1993. To date, there are more than 200 public cord blood banks in the world and even more private ones [25, 26]. In recent years, banks for human cord MSCs and human amniotic epithelial cells (HAECs) have also been established, and these adult stem cell banks will provide an even greater diversity of donor source for organ transplantation or other cell therapy.

7.4 Banking of hES Cells: Dream or Reality?

hES cells possess characteristics that are different from adult stem cells such as the hematopoietic stem cell and the bone marrow mesenchymal stem cells. The hES cells derived from the inner cell mass of the blastocyst can proliferate indefinitely in culture, exhibit a normal karyotype, and are able to differentiate into all cell types under the appropriate conditions [1]. Although some researchers have reported that hES cells and the derived cells or tissues express low levels of HLA molecules [2, 3] and have unique immune-privileged characteristics [27–29], they can express high levels of HLA under certain conditions and therefore allogeneic transplantation with these cells will still require HLA matching [2, 30, 31]. Establishment of a hES cell bank with broad spectrum HLA types will be a necessary solution for obtaining appropriate donors for stem cell therapy [32]. However, the source of these hES cells is part of an ethical debate in some countries as they are obtained from pre-embryos that some argue could otherwise be used for in vitro fertilization-embryo transfer (IVF-ET). An additional problem

is obtaining an adequate number of embryos for establishing the hES cells bank, and the question remains: how many hES cell lines for clinical transplantation would be sufficient for a particular population?

hES cells can differentiate into all cell types under appropriate conditions, and therapies based on these cells for solid organs, such as liver, pancreas, and kidney are theoretically feasible. The HLA matching requirement of solid organ transplantation is not as strict as for hematopoietic stem cell transplantation, and currently it is acceptable in the clinic to use a partially HLA matched donor organ combined with immune suppressive drug treatment. Therefore, hES cell banks may not need as many units as would be required for a bone marrow donor registry or umbilical cord blood bank.

Using a computerized model, Taylor et al. [33] reported on the probabilities of HLA matching 10,000 potential registry donors and 6,500 patients waiting for kidney transplantation. The results showed that 150 donors could serve about 20 % of the recipients with tissues fully matched at the HLA-A, B, and DR loci and about 85 % of the recipients with HLA matched only at the HLA-DR locus. Nakajima et al. [34] also obtained similar conclusions using the same method to analyze HLA and ABO blood group data from a cord blood bank. They estimated that about 170 cell lines could serve 80 % of the patients with 5/6 alleles matched at the 3 loci in the Japanese population. These 2 studies showed that a large but feasible number of cell lines could be banked to serve the majority of the population with HLA matched tissue in a given region. However, there are at least two concerns: (1) whether the embryos from IVF treatment can provide adequate HLA diversity for matching, and (2) whether diversity in the ethnic composition present in different geographical regions influences the number of hES cell lines required for HLA matching.

We established a hES cell bank of an adequate scale using various unwanted embryos from IVF treatment clinics in China and estimated the HLA matching probability of the 174 hES cell lines in the bank with the dataset of 5,236 bone marrow donors in the Hunan branch of the Chinese Marrow Donor Program (CMDP) to evaluate the potential application of this collection [35]. The results showed that the HLA diversity of the cell lines derived from IVF was similar to that of the random sample of the population (Fig. 7.1a). The 174 hES cell lines could serve 24.94 % of assumed patients with full matches at HLA-A, B, and DR loci; 35.14, 56.26, and 49.83 % of patients with a single mismatch at the HLA-A, HLA-B, or HLA-DR locus, respectively; 83.40 % of patients with mismatches at 2 of the HLA-A and HLA-B loci; and 93.45 % of patients with HLA-DR locus matched (Fig. 7.1b). In other words, our bank could serve 24.94–52.65 % of the southern Chinese population with potentially beneficial HLA matching hES cells for organ transplantation or stem cell therapy. The matching probability was close to the theoretical studies described previously [33, 34].

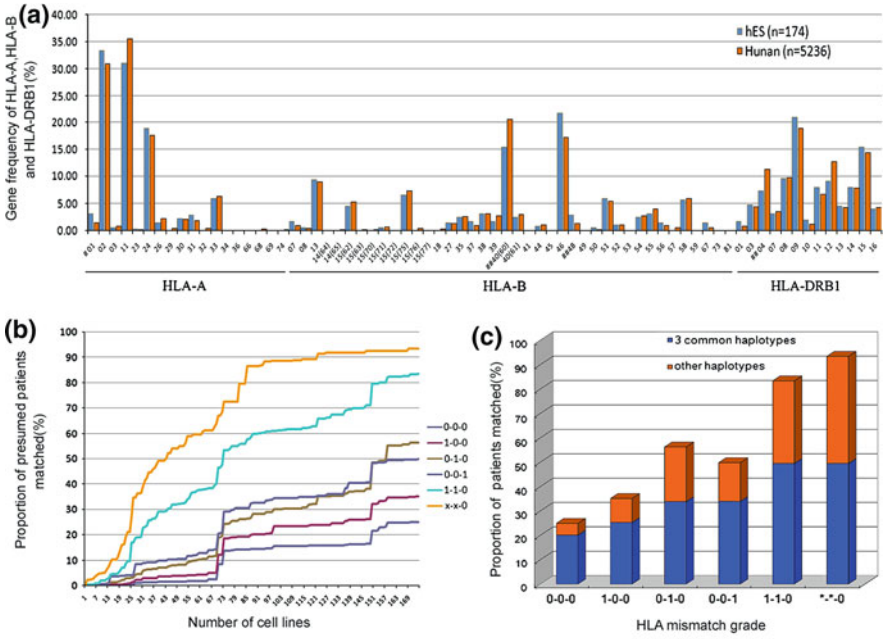

Fig. 7.1 Human leukocyte antigen analysis of 174 hES cell lines and 5,236 individuals from Hunan. **a** Frequency distribution of the human leukocyte associated antigen (*HLA*) genes in 174 hES cell lines and 5,236 individuals from Hunan. Most of the major high-frequency alleles showed similar distribution in the 174 hES cell lines and the 5,236 individuals. Four alleles showed significant difference: # HLA-A*01, $P < 0.01$; * HLA-B*40(60), HLA-B*48, and HLA-DRB1*04, $P < 0.025$. **b** Cumulative percentages of the presumptive patients ($n = 5{,}236$) with an HLA matched donor in the hES cell bank ($n = 174$) at the 6 mismatch levels: 0-0-0, zero HLA-A, HLA-B, and HLA-DR mismatch; 1-0-0, zero HLA-B, and HLA-DR mismatch with a single HLA-A mismatch or better; 0-1-0, zero HLA-A, and HLA-DR mismatch with a single HLA-B mismatch or better; 0-0-1, zero HLA-A, and -B mismatch with a single HLA-DR mismatch or better; 1-1-0, zero HLA-DR mismatch with a single HLA-A and a single HLA-B mismatch or better; *-*-0; zero HLA-DR mismatch. **c** The proportions of the HLA matching rates of the 3 HLA homozygous hES cell lines with common HLA haplotype and the other cell lines in the bank

7.5 "Super-Donors": Donors with Common HLA Haplotypes

HLA-A, HLA-B, and HLA-DRB loci are usually inherited as a linkage unit, or the so-called HLA haplotype. In both theoretical estimations [33] and actual matching analysis [35], a valuable group of donors were identified as "super-donors" for organ transplantation based on their possession of HLA haplotypes that are common in a certain population, and those with blood type O are even more ideal. Unlike heterozygous donors, a homozygous donor can match recipients whether they are homozygous or heterozygous as long as they possess the same HLA haplotype. Therefore, these homozygous "super-donors" can play a significant role in promoting the HLA matching efficiency in a certain population.

In the UK study, it was estimated that 10 homozygous donors with the most common HLA haplotypes could provide a full match for 37.7 % of recipients and a beneficial match for 67.4 % of the recipients [33]. In our 174 established hES cell lines, 8 cell lines are HLA homozygous, which encompass 5 HLA haplotypes. Three hES cell lines share the HLA haplotype of A*02-B*46-DRB1*09, which is the most common HLA haplotype among the 5,236 bone marrow donors representative of the Hunan population. Had any of the three cell lines been found to be blood type O, the total fully matching efficiency would have been increased by 15.9 %. However, the blood types of these hES cell lines are A and B, so the matching efficiency is only increased by 6.4 and 4.0 %. In our hES cell bank, there are two other homozygous hES cell lines with common HLA haplotypes, A*11-B*75-DRB1*12 and A*33-B*58-DRB1*03. Together, the five hES cell lines comprise 80.7 % of the total HLA fully matching efficiency of our hES cell bank (Fig. 7.1c). On the other hand, two homozygous hES cell lines with rare HLA haplotypes A*11-B*35-DRB1*13 and A*33-B*07-DRB1*01 could only provide a 0.23 % matching rate for the local population [35]. The above findings indicate that not all hES cell lines with homozygous HLA haplotypes are super-donors and that only those with common HLA haplotypes and blood type O can provide a high matching rate and significantly reduce the number of hES cell lines needed for banking. Another issue that needs to be considered is the variation of the HLA haplotype frequency in different geographic regions and races. From our summarized data, we could see that the top 6 common HLA haplotypes significantly differ among different geographic regions, different races, and even the same race from different regions (Table 7.1) [36–39]. So the definition of super-donor is variable.

How, therefore, can hES cell banks be established with sufficient numbers of common homozygous HLA haplotypes? The following are some strategies that could be implemented.

7.5.1 Parthenogenic ES Cell Lines

The routine method of parthenogenesis is to activate the oocytes arrested at the second meiosis metaphase and prevent the extrusion of the second polar body. The resulting diploid embryos consist of a genome derived from separated sister chromatids and could be utilized to derive stem cells. This method used to be regarded as a promising way to derive HLA homozygous ES cells [40]. If oocyte donors can be screened for common HLA haplotypes, then hES cell lines with common homozygous HLA haplotypes can be established from any surplus donated oocytes. Nakajima et al. estimated that 80 % of patients are expected to find at least one donor with complete matches at the three HLA loci if homozygous parthenogenic ES cell (pES cell) lines are established from 55 randomly selected donated oocytes [34]. However, it was recently discovered that pES cells are not homozygous in the majority of the genome because of the massive recombination of the genome [41, 42]. It is expected that 70.9 % of human pES cell lines are

Table 7.1 The top 6 common HLA haplotypes in different countries and races

Haplotype	HF%	Haplotype	HF%	Haplotype	HF%
Asia American		*China*		*Japanese*	
A*3303-B*5801g-DRB1*0301	2.86	A*02-B*46-DRB1*09	6.57	A*0206-B*1501-DRB1*1401	12.8
A*3001-B*1302-DRB1*0701	1.64	A*33-B*58-DRB1*03	2.80	A*3101-B*5101-DRB1*1202	8.0
A*0207g-B*4601-DRB1*0901	1.62	A*11-B*15-DRB1*12	2.39	A*2601-B*3901-DRB1*1406	8.0
A*3303-B*4403-DRB1*0701	1.55	A*11-B*13-DRB1*15	2.16	A*0201-B*1501-DRB1*0802	8.0
A*3303-B*5801g-DRB1*1302	1.52	A*11-B*46-DRB1*09	1.86	A*2402-B*3501-DRB1*1401	6.0
A*1101g-B*1502-DRB1*1202	1.37	A*02-B*46-DRB1*08	1.78	A*0201-B*3901-DRB1*1106	4.0
European American		*German*		*France*	
A*0101g-B*0801g-DRB1*0301	7.79	A1-B8-DR3	6.25	A1-B8-DR3	2.48
A*0301g-B*0702g-DRB1*1501	3.54	A3-B7-DR15	3.44	A29-B44-DR7	1.96
A*0201g-B*4402g-DRB1*0401	2.61	A2-B7-DR15	2.23	A3-B7-DR15	1.66
A*0201g-B*0702g-DRB1*1501	2.46	A2-B62-DR4	1.82	A2-B7-DR15	1.21
A*2902-B*4403-DRB1*0701	1.95	A3-B35-DR1	1.70	A2-B44-DR7	0.9
A*0201g-B*1501g-DRB1*0401	1.77	A2-B44-DR4	1.37	A2-B62-DR4	0.9

HF haplotype frequency

HLA heterozygous because of the recombination of homologous chromosomes at the first meiosis [42]. Therefore, most of the pES cell lines derived from oocytes arrested at the second meiotic metaphase are HLA heterozygous [43]. However, haploid mouse embryos from oocytes activated at the second meiotic metaphase with subsequent extrusion of the second polar body can develop into the blastocyst stage and further establish homozygous mouse ES cell lines, automatically becoming diploid [44]. We also observed a similar phenomenon during IVF treatment with the homozygous pES cell line derived from a haploid and spontaneously partheno-activated embryo [45]. Although the exact mechanism and timing of diploidization following oocyte activation is unclear, Revazova et al. successfully derived 4 HLA homozygous pES cell lines with such a strategy [46], providing new methods for increasing the numbers of HLA homozygous pES cell lines.

It is worth noting that there is uncertainty as to whether the differentiation potency and safety of parthenogenic stem cells will be influenced by the abnormal expression of imprinting genes. Although we and other groups showed that human pES cells can differentiate into cells from three germ layers [43, 45–47], research

with chimeric mice suggests the failure of pES cells to differentiate into mesoderm [48], especially to skeletal muscle [49]. Therefore, pES cells need to be fully evaluated for their differentiation potency and safety before being utilized for clinical applications as a source of stem cells.

7.5.2 Induced Pluripotent Stem Cells

Since donors with homozygous common HLA haplotypes from the population can be screened for production of Induced Pluripotent Stem (iPS) cell lines after informed consent, it was suggested to be a more efficient strategy to establish HLA homozygous stem cell banks [50] rather than depending on randomly donated embryos or oocytes. These iPS cells possess the same homozygous HLA typing as the donors. The successful production of iPS cells represented a significant breakthrough in the field of biology. Practically, it is convenient to obtain the source materials for derivation of iPS cells with lower ethical concerns than for hES cells. However, there are some technological barriers that need to be conquered, such as differences in consistency and differentiation potential of iPS cells because of the different extent of reprogramming [51–53]. Furthermore, although iPS cell technology has evolved considerably, the risk of genomic alteration and safety issues need extensive evaluation before iPS cells may be used for clinical applications.

7.5.3 Selection From Unwanted Embryos

The incidence of HLA-homozygous individuals is very low among the common population. According to the cadaveric organ donors reported to the UK Transplant registry, 1.5 % of the population is homozygous at HLA-A, HLA-B, and HLA-DR [33], while 1.1 % are such homozygotes in the Chinese population according to the data from the Hunan bone marrow bank. In our hES cell bank, the frequency of HLA homozygotes among the hES cell lines derived from "unwanted" IVF-ET embryos is 4.1 % [35], higher than that of the common population. The reason for this difference is unclear, but it indicates nevertheless that many "unwanted" embryos discarded during IVF-ET cycles can be an important source for deriving hES cell lines homozygous for common HLA haplotypes.

7.6 Clinical Standards for Banking

One of the key problems for the application of stem cell research is determining how to establish a hES cell bank to meet the clinical criteria. Although a basic consensus is the whole production process must meet the Current Good Manufacture Practices

(cGMP) standards and local rules and regulations, there are no uniform standards internationally to regulate the generation of clinical-grade hES cell lines. In this chapter, we explore this issue from the following five aspects.

7.6.1 Informed Consent and Medical History Collection of Donors

The IVF patients who consent to donate their surplus embryos or gametes for stem cell research have the right to know and agree to the purpose of the research. The researchers must provide all the information required for the donors to make the decision, including aim and significance, how their embryos or gametes will be used, as well as the respective rights and obligations of donors and researchers in the research. That is, the researchers must present details of the research and answer the donors' questions patiently in order for them to fully understand the pros and cons of donation and to make the decision independently, voluntarily, and rationally. The donors are accountable for their donation under informed consent. Of note, these individuals are not paid for their donations in order to avoid misconduct and any conflict of interests. National Institutes of Health (NIH) even require certain types of informed consent forms showing that cells were first freely donated for scientific research generally and then for stem cell research specifically [54].

Besides the ethical conduct in recruitment of the donors, the related medical records of donors, including family medical history, genetic disease, occupational risks, and other medical history, should be collected anonymously with informed consent. It is necessary to screen all the IVF pre-treatment examination data including tests for sexually-transmitted diseases and other infectious diseases (hepatitis B virus, hepatitis C virus, human immunodeficiency virus, syphilis, Chlamydia, mycoplasma, cytomegalovirus, toxoplasma, rubella virus, etc.), chest X-ray (for tuberculosis), and karyotyping. Only the embryos donated by healthy individuals can be chosen for the derivation of the stem cell lines for potential future clinical applications. It may also be necessary to collect and store the blood samples of donors for newly developed tests in the future.

7.6.2 Embryo Source for hES Cell Derivation

Which type of embryos should be selected for the derivation of hES cells? In our opinion, high-quality frozen embryos, evaluated according to morphology assessment, are the best choice for derivation. These viable embryos have less likelihood of being aneuploid [55, 56], and the majority of the resulting hES cell lines have normal karyotype. However, are the high-quality embryos the only source for the derivation of clinical-grade hES cell lines? Normal embryos have the potential to develop into a human being when successfully implanted, so the

destruction of large numbers of normal human embryos to obtain cell lines is a point of ethical controversy. To avoid this problem, researchers have made many attempts to search for new sources and methods for the establishment of hES cell lines.

First, the large number of abandoned embryos that are unsuitable for IVF treatment may provide a source for derivation of hES cell lines with less ethical concerns. These embryos are abandoned because of poor-quality or abnormal fertilization. Several studies showed that these "unwanted" embryos could be an important source of normal hES cell lines [57–62]. Lerou [59] reported that early-arrested or highly fragmented embryos which have achieved the blastocyst stage are a robust source of normal hES cells. In our study, the derivation efficiency using "unwanted" embryos (including poor-quality and abnormally fertilized embryos) was 27.2 %, while that for blastocysts with no obvious inner cell mass was 7.2 %. Moreover, 104 hES cell lines were derived from 344 abnormally fertilized blastocysts, and among them only 19 cell lines displayed abnormal karyotypes. Only one cell line isolated from 52 mono-pronuclear (1PN) zygotes showed karyotypic abnormalities; meanwhile, half (15/30) of the cell lines from tri-pronuclear (3PN) zygotes showed normal karyotypes, although the abnormality rate of 3PN zygotes was significantly higher than that of other abnormally fertilized groups [35]. This indicates that the embryos discarded because of various abnormalities are valuable resources for establishing hES cells with normal karyotypes. In reality, there is a great number of such embryos produced during the IVF treatment. For example, in our collaborative IVF center, there were 7,939 IVF cycles in 2009 which generated a total of 10,519 "unwanted" embryos. In our survey, more than half of the patients are willing to donate their "unwanted" embryos for research purposes, so thousands of embryos would potentially be available for hES cell derivation.

Another alternative is to derive hES cell lines from a single blastomere. Several reports have proven that a single blastomere from 4 $\sim$ 8-cell pre-implantation embryos could be used for hES cell derivation [63–65]. This technology is established upon embryo biopsy. When the embryo reaches the 4 $\sim$ 8-cell stage, one or two blastomeres are removed and cultured until transformed into hES cells. This method does not interfere with the developmental capacity of the parent embryo. A major short-coming of this method, however, is the low efficiency since less than 2 % of the single blastomeres will ultimately form hES cells [65].

No matter which type of embryo source is used for establishment of hES cell lines, the quality needs to be observed and the developmental conditions recorded. These parameters include fertilization type, quality of the embryos and karyotype of the resulting hES cells. At present, karyotyping is a common test for the genomic integrity of hES cell lines derived from various embryos. However, there are many types of slight genetic abnormalities and chimerism in the IVF embryos [66], and therefore it is necessary to apply more precise methods, such as array comparative genomic hybridization (array-CGH) or single nucleotide polymorphism (SNP) chips to analyze the genetic integrity of hES cells, especially those derived from abnormal or poor-quality embryos.

7.6.3 Clinically Applicable Cell Production Technology

Stem cell-based products for clinical applications are required to be produced according to cGMP guidelines. The cGMP requirements for the derivation and culture of hES cell lines have been systematically discussed [67, 68], and the generation of clinical-grade hES cell lines has also been reported in 2007 [69]. The major efforts being made for a clinically-applicable methodology is using human feeder cells to replace the mouse feeder cells and serum replacement (SR) substituting for fetal bovine serum to eliminate materials of animal origin [69–74]. These improvements avoid the risk of cross-contamination with xenogeneic pathogens and immunogens, such as Neu5Gc [75]. At present, human feeder cells and xeno-free SR manufactured under GMP conditions are commercially available but these humanized culture conditions still have the risk of contamination with human pathogens, and expensive pathogen screening assays are required for the final products. An ideal culture system will be feeder free using chemically-defined culture medium combined with synthesized extracellular matrix. mTeSR [76, 77] and StemPro hES cell SFM [78] are two commercially available chemically-defined media which have the capacity to maintain long-term and stable expansion of hES cells. Only mTeSR was reported to support successful derivation of two new hES cell lines when combined with recombinant extracellular matrix, however, both of them subsequently turned out to be karyotypically abnormal [76]. It is possible, therefore, that this culture system will cause genomic instability after long-term culture. Up until now, there have been no other reports of the successful derivation of hES cell lines using chemically-defined medium. Therefore, the current chemically-defined culture conditions need further optimization.

The ability to generate human stem cell-derived cell types in sufficiently high numbers is essential for clinical application. Taking islet transplantation for a type 1 diabetes mellitus as an example, 11,000 islets per kg of body weight are needed for a patient [79], that is over 1 billion beta cells per transplant for a 70 kg patient. The number of hES cells cultured as colonies to full confluency is about 10 million per 6-well plate, and often only a small percentage of the cells can differentiate into the desired cell types. Except for the further improvement of the differentiation efficiency, an automated and scale-up culture method is also needed. Currently used manual dissection or enzyme digestion for passaging hES cells in culture dishes no longer meet the demand for future clinical applications. The development of scalable hES cell culture systems is still in its infancy, but has already made promising progress. Using a stirred-suspension bioreactor culture, several scale-up culture protocols have been established and proved much more efficient than routine culture methods [80–84]. In addition, this large-scale culture system could be adapted to direct differentiation of hES cells into neural, endodermal, and cardiac cells [84–87]. Further analysis will be needed to address whether this large-scale expansion and differentiation method could maintain the genomic integrity and normal function of differentiated cells.

7.6.4 Characterization of hES Cell Lines and Quality Control of Cell Banking

Any newly derived hES cell line should be fully characterized according to the well-known stem cell properties, including the expression of hES cell-specific markers and pluripotency-related genes, karyotyping, telomerase activity, and its differentiation potency in vitro and in vivo. Furthermore, some specific tests for cell banking should be performed, such as DNA fingerprinting for identification of each cell line. HLA and ABO typing should also be performed for tissue matching in potential future transplantation therapy. Proper screening for pathogens is also essential before cell storage and as well as for therapeutic application.

One of the concerns of stem cell clinical applications is the safety of the cell products themselves. hES cells can acquire genetic and epigenetic changes after prolonged culture [88–97], which lead to an enhanced proliferation and anti-apoptotic capability and even some features of neoplastic progression [88, 95, 98, 99]. Chakravarti and colleagues assessed the genomic fidelity of paired early- and late-passage hES cell lines. They found that the late-passage cells acquire complex genomic alterations including aberrations in copy number (45 %), mitochondrial DNA sequence (22 %), and gene promoter methylation (90 %), which are commonly observed in human cancers [94]. Therefore, it will be important to establish comprehensive and sensitive screening methods to monitor any genetic and epigenetic changes during long-term culture. And further studies are needed to identify which type and to what extent the genetic and epigenetic changes are of biological significance.

7.7 Personalized Treatment Versus Banking

The best way to avoid immune rejection is personalized treatment using the patients' own cells. For transplantation therapy based on pluripotent stem cells, the ideal strategy would include reprogramming the patients' own somatic cells by somatic cell nuclear transfer (SCNT) or iPS methods to the pluripotent state [100–102] or directly transforming them into a functional cell type required for the treatment [103, 104]. Although SCNT has not succeeded for humans, reprogramming technologies based on transgenes or protein delivery is fast developing and has caused a public debate on the necessity of banking stem cells. In our opinion, banking is necessary. Although the full-term development capacity of iPS cells has been proven recently [105, 106], the efficiency with which full-term mice can be produced from iPS cell-complemented tetraploid blastocysts is very low. This indicates that the current iPS cell technology is inefficient to achieve full reprogramming. It has also been reported that iPS cells and hES cells differ considerably in their gene expression profile [107, 108]. Therefore, iPS cells are not identical to hES cells. Besides, immune rejection is not the only barrier to successful stem cell therapy: it is equally important to know

how to direct differentiation of stem cells into the functional cells required for treatment, as well as how to ensure safe therapy from human pluripotent stem cells. It has, for instance, been shown that there are marked differences in differentiation [109] or tumorigenicity between hES cell lines and iPS cell lines [110]. Differences exist even among those iPS cell lines generated from the same source [102, 109]. Before the possible reasons and the countermeasures can be discovered, it will be difficult to ensure the efficiency and sufficiency of iPS cells reprogrammed from skin cells for clinical therapy. Furthermore, for older individuals, although iPS cells can be reprogrammed from their own somatic cells, activation of additional genes such as TERT [111], related to maintenance of high cell proliferation, may be required, which raises extra safety concerns. As for the patients with genetic disorders, their somatic cells carrying genetic defects are not suitable to be reprogrammed and re-differentiated for treatment. Although reprogramming combined with gene modification strategies have been tested in the mouse model with genetic disorders [112–114], using gene therapy poses additional risks that must be considered in the human clinical setting.

From an industrial perspective, personalized treatment is not cost-effective because lots of expensive and labor-intensive quality control tests are needed for each individualized cellular product. In contrast, banking of hES cells, especially from "super-donors", provides the possibility for the industry to produce far fewer batches of off-the-shelf stem cell products that will benefit millions of people. Therefore, in effect, hES cell banking remains indispensable for the future of regenerative medicine.

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Chapter 8
Generation of Histocompatible Tissues via Parthenogenesis

Paula W. Lampton, Judith A. Newmark and Ann A. Kiessling

Abstract Parthenogenic stem (PS) cells are a potential source of histocompatible, pluripotent cells for transplantation therapy that does not rely on fertilized embryos. Parthenotes result from artificially activated oocytes that contain only maternal chromosomes, without contribution from sperm. Parthenotes cannot develop into live offspring, but have been used to derive PS cells in multiple species, including mouse and humans. Different oocyte activation protocols and natural recombination events may lead to PS cells that are heterozygous at the major histocompatibility complex (MHC) and therefore completely histocompatible to the oocyte donor, or MHC-homozygous and histocompatible to a significant percentage of the general population. Studies in mouse and nonhuman primates suggest the PS cells may be a valuable cell source for transplantation therapies, although further work is needed to establish the safety and efficacy of human PS cell-based therapies.

8.1 Introduction

Parthenogenesis is a mechanism by which oocytes (eggs) initiate development without the contribution of paternal chromosomes derived from sperm. Oocytes are activated artificially using a chemical or a physical signal as opposed to the joining of a male germ cell (sperm) with a female germ cell (oocyte, egg). Artificially

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activated eggs are termed parthenotes; they develop normally through several early stages, but arrest by midgestation and therefore are not capable of developing into a complete organism. Thus, parthenotes provide a source of material for research or therapeutic purposes that do not possess the same ethical constraints of developing embryos. The pluripotent cells of a parthenote can be isolated to produce a parthenogenic stem (PS) cell line containing characteristics similar to a conventional fertilized embryonic stem (ES) cell line. PS cells are a source of pluripotent cells that may be used in transplantation therapy to repair or to replace tissues that have been damaged due to injury or disease. PS cells are sometimes referred to as parthenogenic embryonic stem (pES) cells, but we have adopted the more accurate nomenclature of PS cells. In this chapter, we will discuss the process of parthenogenesis in mammalian systems and how it has been harnessed to develop pluripotent cell lines. We will also discuss the potential uses of PS cells in transplantation therapy and their potential for overcoming the issues concerning histoincompatibility that currently hinder pluripotent stem cell therapy.

8.2 Parthenogenesis

8.2.1 Parthenogenic Activation

Parthenogenesis, or activation of the developmental pathway in an unfertilized oocyte, is a form of asexual reproduction that occurs naturally in many lower species. In mammalian species, parthenogenesis can occur spontaneously in vivo and can lead to the production of dermoid cysts or teratomas but it does not lead to the development of offspring. In vitro parthenogenesis is stimulated by applying a chemical or a physical cue to substitute for the natural fertilization of an oocyte by a sperm. “Parthenote” is the term used to describe the artificially activated egg; various methods of producing parthenotes are illustrated in Fig. 8.1. Although an enucleated oocyte may be used to produce an androgenic embryo that uses only the paternal chromosomes (Fig. 8.1a), parthenote refers to the activation of an oocyte to produce an “embryo” which is derived using only the maternal chromosomes. A gynogenote contains maternal genomes from two different oocytes (Fig. 8.1b), whereas a conventional parthenote contains the maternal genome of one oocyte in either haploid or diploid form (Fig. 8.1c). The parthenotes referred to in this chapter for the derivation of pluripotent stem cells were obtained using the method described in Fig. 8.1c.

Parthenogenic preimplantation development is similar to conventional embryonic preimplantation development. A fully mature egg is produced by meiosis, a two-stage form of reductional division. During oocyte growth and maturation, the chromosomes are duplicated (4n) and recombination of the alleles occurs. In the first stage of meiosis (MI), the first set (2n) of chromosomes is expelled in the first polar body, a markedly asymmetric cell division that leaves the large (100–120 μm)

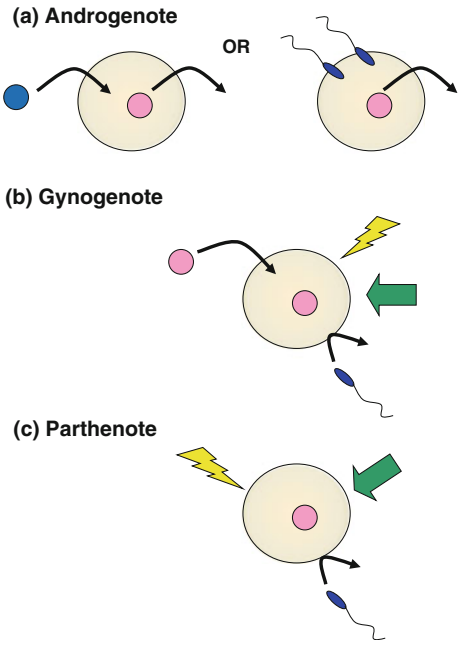

Fig. 8.1 Parthenogenic activation. **a** An androgenote is formed when an enucleated oocyte is fertilized by male pronuclei or two sperm (in *blue*). **b** A gynogenote is formed when an oocyte receives another maternal genome followed by electrical (*green*), chemical (*yellow*), or sperm-based activation such that there is no paternal integration of the genome. **c** A conventional parthenote is formed when an oocyte is artificially activated by electrical, chemical, or sperm-based signals

egg with a diploid set of chromosomes comprised of gene alleles from both parents. Upon activation by sperm penetration or an artificial stimulus, the second stage of meiosis (MII) occurs and one chromosome of each pair is expelled in a second polar body leaving a haploid (1n) egg. Suppression of the extrusion of polar bodies may be used during parthenogenic stimulation to maintain the diploid state of the egg. Recombination events during meiosis may result in the resulting parthenotes being heterozygous in regions of the genome. Other protocols allow haploid parthenotes to form and rely on spontaneous diploidization of the genome during PS cell generation, which should result in a fully homozygous PS cell line since recombination has already occurred.

After activation, the egg undergoes a series of cleavage divisions in which the overall size of the parthenote remains the same while the individual cells (blastomeres) become smaller in size. At about day 4 of development, approximately the 16-cell stage, the first cell commitment occurs as one or two cells become trapped in the middle, and the outer cells form membrane junctions in a process termed compaction. Expression of the genes responsible for water transport into the interior of the cell mass creates a fluid-filled blastocyst with the inner cells located together at one end. The outer cells of the blastocyst (trophectoderm) will become the placenta, while the inner cells will form the inner cell mass (ICM), which will continue to form the fetus. The fetus from a fertilized embryo continues to develop to term, while parthenotes arrest at midgestation.

8.2.2 Developmental Potential of Parthenotes

Mice have a normal gestation period of 18–21 days, while in humans gestation takes 38–42 weeks. Mouse parthenotes generally arrest their development and die by day 10. The reason for this developmental arrest lies within the phenomenon of imprinting, whereby certain genes are preferentially or exclusively expressed by either the maternal or paternal chromosomes. Imprinting is typically controlled by methylation of specific DNA residues during gametogenesis. Parthenotes lack paternal genes and therefore show inappropriate expression of genes such as *Dlk*1, *Igf*2, and *H*19 [1]. Parthenotes typically display repressed levels of paternally imprinted genes, whereas the levels of maternally imprinted genes are doubled. Development to term and to adulthood has been achieved in mice with only maternal chromosomes by altering the maternally imprinted H19 gene, and thus generating a gene expression pattern more typical of fertilized embryos [2, 3]. The role of imprinting in PS cells is discussed in greater detail in Sect. 8.4.3. Another method to overcome imprinting issues utilized egg chromosomes from immature oocytes for fertilization instead of a sperm, since these have incomplete maternal imprinting with somewhat repressed maternal gene expression and somewhat activated paternal gene expression [4]. The resulting gynogenote "embryos" were used to generate pluripotent stem cells; however their developmental potential to produce live offspring was not tested.

8.2.3 Oocytes for Parthenogenic Activation

Oocytes for mouse parthenogenic research are plentiful, however, there are several barriers to human parthenogenic research. Although the use of oocytes as opposed to embryos for creating pluripotent stem cell lines addresses the moral issue involved in depriving an organism of life, there are other moral issues to address. One dilemma concerns the medical protocol used to collect oocytes from healthy women. Curiously, the debate about women donating eggs for research purposes has not allowed the standard guidelines used by human subjects research committees to determine safety procedures for the women and the research. Instead, some groups concerned with the exploitation of women have argued that for their protection, women donating eggs for research purposes should not be monetarily compensated for their participation in the research project. This is a highly unusual circumstance because normal human subjects who volunteer for other biomedical research projects are compensated for time and effort expended on the research according to guidelines established by institutional human subjects review committees, in compliance with international guidelines for biomedical research, such as the Belmont Report. The guidelines ensure that monetary inducement does not lead to risk taking by circumstances such as non-disclosure of pre-existing medical conditions [5]. Nonetheless, rather than leave protection of the research egg donors to

standing research review committees, some states, such as California, have prohibitions against monetary compensation of egg donors included in stem cell legislation (www.cirm.ca.gov). In response to the confusion surrounding this issue, other states, such as New York, have enacted legislation that specifically allows standard monetary compensation for egg donors for research purposes (stemcell.ny.gov/oocyte_donation.html). Other research oversight groups, such as the Human Fertilization and Embryology Authority in England have developed "egg sharing" guidelines that allow fertility clinics to lower the cost of fertility treatments for women willing to donate some of their eggs for research purposes [6].

The rancor surrounding the debates about egg donation for research purposes are based in part on concerns about the vigorous hormone stimulation given to women undergoing infertility treatment, and to women who donate eggs to other women for fertility treatment. To address these concerns, the National Academy of Sciences convened a conference of experts in hormone stimulation and hormone-responsive diseases of women, such as ovarian, breast, and endometrial cancers [7]. The consensus report highlighted the low level of morbidity for the hundreds of thousands of women who have undergone hormonal stimulation for assisted reproduction, but acknowledged that the long-term consequences (greater than 32 years) of such treatment are not yet known. Overall, these barriers have led to a dearth of human oocytes for parthenogenic research.

8.3 Derivation of PS Cells

8.3.1 Parthenogenic Cell Lines

ES cell lines are generated from blastocyst stage embryos; hence parthenogenic cell lines are generated from parthenogenic blastocysts. The first mouse PS cell line was generated in 1983 from haploid oocytes, activated by ethanol, which underwent diploidization during PS cell derivation to create homozygous parthenote cell lines [8]. A variety of mouse PS cell lines using different derivation procedures have since been generated and studied [9–11].

The first primate PS cell line, Cyno-1, was generated in 2002 from *Macaca fascicularis* [12, 13], and was followed by the generation of five more parthenote cell lines from the rhesus monkey *Macaca mulatta* [14]. The first human PS cell line was created in 2004 during a failed attempt at creating nuclear transfer ES cell lines, but it was not authenticated as such until 2007 [15–17]. Since that time, at least 15 more human parthenote (hPS) cell lines have been generated and used in research [18–22].

8.3.2 Derivation of PS Cell Lines

There are a variety of methods that may be used to generate PS cells, several of which are summarized by Cibelli et al. [23]. A number of electrical or chemical stimuli are capable of inducing parthenogenesis by triggering calcium oscillations and releasing the oocytes from metaphase II arrest. Chemical stimuli include ethanol, calcium ionophore, ionomycin, and strontium, depending on the species and chosen protocol. It is necessary to include an inhibitor of protein synthesis such as 6-dimethylaminopurine (6-DMAP) to preclude the oocytes from reentering cell cycle arrest [24]. Activation of oocytes may lead to haploid cells if the second polar bodies are extruded from the oocyte. However, blocks such as cytochalasin B may be used to prevent this extrusion, thus creating diploid embryos. It has been reported that during the early passages of mouse haploid parthenote cell growth, many cell lines undergo diploidization spontaneously [8].

Several factors are important in generating a PS cell line. The choice of culture medium or oxygen concentration (low is preferable) are important [20]. Concentrations of the activation agents or other drugs must be optimized as well as the incubation times. Variations in these conditions may lead to significant differences in calcium oscillations within the egg cytoplasm and, therefore, in the potential of the resulting parthenotes [25]. The most important factor in generating PS cells is arguably the issue of timing of egg activation, which determines the zygosity and recombination signature of the resulting cell line. The next section will discuss the issue of recombination in producing homozygous and heterozygous PS cell lines. Figure 8.2 illustrates a comparison of two types of parthenogenesis compared to conventional fertilization. Figure 8.2a illustrates the biparental nature of a conventionally fertilized oocyte. MII PS cells ("p(MII) ES") cells are created by allowing extrusion of the first polar body and then suppressing the second polar body at the time of oocyte activation (Fig. 8.1b). This results in a diploid cell line with chromosomes remaining after extrusion of the first polar body. Alternatively, suppression of the first polar body, followed by egg activation that allows polar body extrusion ("p(MI) ES") results in diploid MI PS cells with a different distribution of recombined chromosomes (Fig. 8.1c). Both methods may be used to successfully create mouse PS cells completely histocompatible with the oocyte donor [11].

8.3.3 MHC/HLA Heterozygous and Homozygous PS Cells

Tissue matching of the major histocompatibility complex (MHC), which is known as the human leukocyte-associated antigen (HLA) complex in humans, is crucial to any form of transplantation therapy. During oocyte maturation, recombination of the chromosomes occurs. Recombination may occur anywhere within the genome, but recombination of the MHC determines whether the resulting PS cells are

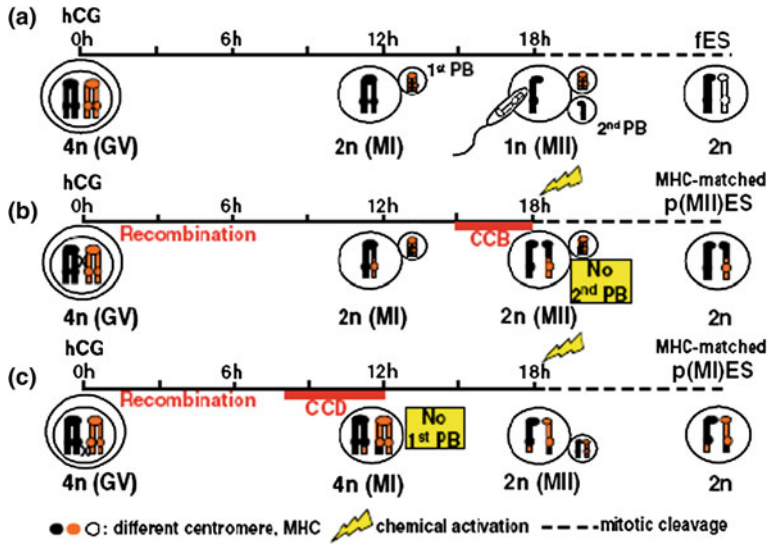

Fig. 8.2 Comparison of the process to create fertilized ES cells with two methods for generating PS cells. Germinal vesicle (GV) stage oocytes are matured in culture and may undergo genomic recombination. **a** Fertilized ES cells form when an MII stage oocyte is fertilized by a sperm followed by extrusion of the second polar body and mitotic cleavage. **b** p(MII)ES cells are generated by artificial activation of an MII oocyte with the simultaneous use of cytochalasin B (CCB) to prevent extrusion of the second polar body. **c** p(MI)ES cells are generated by using cytochalasin D (CCD) to prevent extrusion of the first polar body followed by activation, extrusion of the second polar body, and mitotic cleavage. From Ref. [11]. Reprinted with permission from AAAS

MHC-homozygous (no recombination of the MHC) or heterozygous (MHC recombination has occurred). MI PS and MII PS cells can be either homozygous or heterozygous at the MHC. There are advantages and disadvantages to MHC-heterozygous and MHC-homozygous PS cells, which will be discussed in more detail in Sect. 8.5.2. Lin et al. [18] were able to generate a highly homozygous human PS cell line, and Revazova et al. [21] generated four HLA homozygous human PS cell lines by using two approaches. The first approach used HLA homozygous individuals as oocyte donors. However, HLA homozygous individuals are extremely rare. The second approach used HLA heterozygous individuals as oocyte donors, but utilized a fourth method for creating a parthenote that utilized calcium ionophore along with puromycin (instead of 6-DMAP) to create haploid parthenote blastomeres. The resulting PS cells derived using this approach, were diploid (presumably by chromosome duplication) and fully homozygous, including the HLA region.

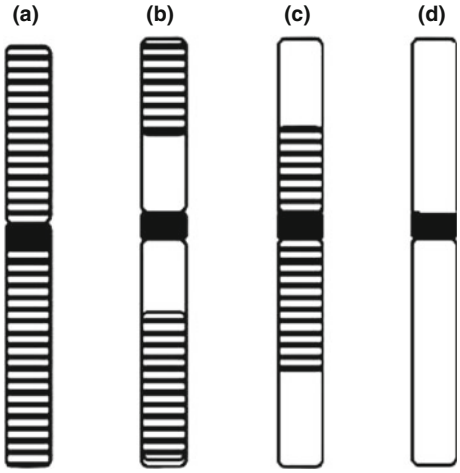

Fig. 8.3 Genome-wide single nucleotide polymorphism (SNP) analysis measurement of heterozygosity. Fertilized ES cells show high levels of heterozygosity (**a**). p(MII)ES cells show heterozygosity toward the telomeres (**b**), whereas p(MI)ES cells show heterozygosity near the centromere (**c**). Fully homozygous ES cells show no heterozygosity (**d**). Panels **a**–**c** are reprinted from Ref. [16], with permission from Elsevier. (Panel **d** is original to this chapter.)

8.3.4 Recombination Signature of PS Cells

PS cell lines contain unique recombination signatures that can be analyzed by genome-wide single nucleotide polymorphism (SNP) analysis. Figure 8.3 shows the distribution of heterozygosity that is associated with fertilized ES cells, MII PS, MI PS, and homozygous PS cell lines. This technique was elegantly applied to the authentication of the first human PS cell line, which emulates the predicted pattern of heterozygosity for a p(MII)ES cell line [17]. Heterozygosity by SNP analysis across the genome is measured with respect to the distance from the centromere. Random heterozygosity, such as the pattern displayed in Fig. 8.3a, was found to be associated with conventional fertilization or with nuclear transfer-derived embryos and displays no relationship to the centromere. In contrast, PS cells displayed distinct patterns of heterozygosity that were related to the distance from the centromere and were correlated with stage of meiosis that was interrupted to produce the PS cells. MII PS cells showed heterozygosity toward the telomeres (Fig. 8.3b), whereas MI PS cells showed heterozygosity close to the centromere (Fig. 8.3c). PS cells that are fully homozygous across the genome would have no heterozygous regions (Fig. 8.3d). Thus, a combination of HLA genotyping and SNP analysis can be used to determine how suitably matched a PS cell line is to a potential patient.

8.4 Developmental Potential of PS cells

Despite the inability of parthenote embryos to develop to term, PS cells exhibit remarkable pluripotency. This section will examine the ability of PS cells to differentiate into various cell types and lineages, both in vitro and in vivo (in animal models). The impact of global and imprinted gene expression in PS cells compared to stem cells derived from fertilized embryos (ES cells) will also be analyzed in relation to pluripotency.

8.4.1 Differentiation Potential of Mouse PS Cells

Multiple studies on early mouse PS cells extensively analyzed the ability of these cells to differentiate into multiple cells types [8, 9]. These studies report the ability of PS cells to differentiate in vitro into embryoid bodies, including markers from the three embryonic germ layers [9]. Later analyses of both "p(MI)ES" and "p(MII)ES" cells support their ability to differentiate in vitro into cells from all three germ layers, including cardiomyocytes and cells of hematopoietic lineage [11, 26, 27].

In vivo, mouse PS cells developed into teratomas when injected into immunodeficient mice. Allen et al. [9] found that PS cells were limited in their ability to differentiate into skeletal muscle in teratomas (0–5 % skeletal muscle, compared to 25 % in control ES cells). Delayed myogenic differentiation and expression of myogenic transcripts of PS cells was confirmed by a separate group of researchers [28]. However, other studies have shown skeletal muscle differentiation in teratomas from PS cells [11, 26, 29].

Allen et al. [9] produced the first comprehensive analysis of PS cell contribution to chimeric mice and tetraploid embryos. PS cells were able to contribute to chimeric mice (ranging from 5 to 70 %) and showed normal prenatal and postnatal growth rates, compared to obvious growth retardation in chimeras from parthenote four-cell stage blastomeres. However, in this study the PS cells did not significantly contribute to skeletal muscle cells in the chimeric mice, in contrast to normal ES cells and similar to their results in teratomas. More recently, mouse PS cells were able to effectively contribute to skeletal muscle tissues in chimeric mice [29]. It is tempting to speculate that the recently reported likelihood of aneuploidy in early cleavage stage blastomeres [30] may also be true for parthenote blastomeres, a situation that appears to be corrected in the ICM cells from which stem cells are derived.

Tetraploid embryo complementation (TEC) has also been utilized to analyze the in vivo differentiation potential of PS cells. In this technique, ES cells are injected into a tetraploid mouse embryo; the tetraploid embryo provides the placental support, while the developing fetus is derived solely from the ES cells. Tetraploid embryos implant with high efficiency, but do not normally develop into

a fetus. However, the tetraploid embryo may be "complemented" with pluripotent cells such as ES cells [31]. Multiple groups have utilized this technique but were not able to achieve full prenatal development with PS cells, despite success with normal ES cells [9, 11, 32]. Most recently, Chen et al. [29] were able to produce three live, anatomically normal pups through TEC using PS cells. Although these mice died shortly after birth, this is further evidence that PS cells are pluripotent.

The in vivo differentiation studies of mouse PS cells point to possible differences in differentiation potential between cell lines. These apparent discrepancies may be due to differences in oocyte activation protocols and passage number of ES cells utilized for the in vivo differentiation studies. Chen et al. [29] utilized strontium for oocyte activation and found improved PS cell contribution to chimeras when utilizing midpassage ES cells [33]. In contrast, Allen et al. [9] utilized ethanol for activation and used very early passages for their studies in which limited differentiation into skeletal muscle tissue was found. These data will likely be useful for evaluating therapeutic potential of new and existing PS cell lines.

8.4.2 Differentiation Potential of Human and Nonhuman Primate PS Cells

Like mouse PS cells, human and nonhuman primate PS cells appear to be pluripotent and capable of differentiation into all cell types. PS cells (Cyno-1) derived from parthenote monkey embryos (*Macaca fascicularis*) have been shown to differentiate into numerous cell types in vitro, as well as form teratomas when injected into immunodeficient mice. Cyno-1 cells have been well characterized for their ability to generate nestin-positive neural precursors, a potentially useful cell type for transplantation therapy, which will be discussed in further detail in Sect. 8.5.3.

Human PS cells have also been recently described and initially characterized for their differentiation potential. Revazova et al. [20, 21] showed that various human PS cell lines could differentiate into cells from the three germ layers, both in vitro and in vivo through teratomas. Brevini et al. [22] also reported the derivation and in vitro differentiation of human PS cells. However, with these human PS cell lines, in vivo differentiation via teratomas was either poor or resulted in tumor formation. Altered expression of genes involved in mitotic spindle formation and spindle check points was found in these cell lines and proposed as a cause of tumor formation [22].

Research with human PS cells is currently limited to private funding in the United States, and therefore the data on differentiated cell types from human PS cells are limited (www.bedfordresearch.org). The International Stem Cell Corporation has reported the differentiation of cells from human PS cells that are potentially useful for transplantation therapy, including retinal pigmented epithelial cells, but these data are not yet published. The evidence that PS cells from

multiple species are pluripotent is extremely promising for the use of these cells to generate tissues useful for transplantation therapy. The existing data on parthenogenic tissues in transplantation research will be discussed in Sect. 8.5.

8.4.3 Imprinting and Gene Expression in PS Cells

The inability of parthenote embryos to develop to term is likely due in large part to errors in imprinted gene expression that are crucial for embryonic and extraembryonic development. Several paternally imprinted genes are expressed only by male alleles during embryonic development and are not expressed in parthenotes. Therefore, the expression of imprinted genes in PS cells and its impact on stem cell pluripotency is an area of major concern.

Despite the established imprinting errors in parthenotes, there is increasing evidence pointing to the ability of PS cells to reprogram imprinted gene expression. Jiang et al. [26] detected expression of seven paternally imprinted genes in their newly derived mouse PS cell line, although expression levels of some of these genes were reduced compared to fertilized ES cell controls. At least three of these genes (*Snrpn, Mest*, and *Peg*3) were detected in both fertilized and parthenogenic blastocysts, suggesting that some reprogramming occurs even during embryonic development.

In another study, Li et al. [33] analyzed the expression of imprinted genes in parthenotes and PS cells as well as PS cell fetuses obtained through tetraploid embryo complementation (PS-TEC). They found that many paternally imprinted genes analyzed were silenced in parthenogenic blastocysts, but all analyzed paternally imprinted genes were activated in PS cells. The reprogramming of imprinted gene expression in PS cells is correlated with isolation and in vitro culture of PS cells as well as DNA methylation patterns [26, 33]. Differences in global methylation patterns and in methylation of some differentially methylated regions (DMRs) were found between PS cells and ES cells. However, methylation of DMRs that control expression of several paternally imprinted genes, such as *Snrpn*, *Peg*1, and *U2af* 1-*rs*1, appears to be reprogrammed in these mouse PS cells [33]. Therefore, these recent studies strongly suggest that the culture and isolation of PS cells incompletely, but significantly, reprograms paternally expressed imprinted genes.

Differences in imprinted gene expression likely exist between different mouse PS cell lines and different passages within a cell line [9, 26, 33]. However, non-imprinted gene expression patterns differ even between fertilized ES cell lines. Differences in gene expression between mouse ES and PS cell lines appears to lie within the normal range of deviation between ES cell lines [34]. Furthermore, ES cells have also been shown to have some epigenetic instability and errors in imprinting, yet are still considered a viable cell source for transplantation therapy [35]. Although imprinted genes clearly affect embryonic development in vivo, the impact of altered imprinted gene expression in differentiated parthenogenic cells

has not yet been determined. Detailed analyses of individual cell lines and differentiated cell types from PS cell lines will clearly be necessary before these cell lines may be used for therapeutic purposes. The next section of this chapter will evaluate the ability of PS cell derivatives to overcome histoincompatibility in transplantation therapy.

8.5 Immunogenicity and Therapeutic Potential of PS Cells

8.5.1 Immunogenicity of ES Cells

During allogeneic tissue transplantation, polymorphic molecules of the MHC, which normally serve to alert T cells to the presence of foreign antigens, will trigger an immune response against the graft tissue. To aid transplantation success, the MHC alleles (HLA in humans) of potential organ donors and the organ recipient are analyzed to provide the highest number of allelic MHC matches. Since pluripotent stem cells may be differentiated into cell types useful for transplantation therapy, the immunogenicity of these cells has been an area of increasing research.

ES cells and their derivatives seem to possess unique immunological properties compared to somatic cells. In mouse and human embryonic stem (hES) cells, the level of MHC class I molecules is lower than in typical somatic cells. The T cell response against hES cells was also greatly diminished in comparison to somatic cells (reviewed in [36]). All evidence to date has shown that PS cells exhibit similar expression of MHC proteins and MHC chaperone molecules compared to fertilized ES cells [11, 37]. Therefore, research on the immune response of ES cell-derived tissues for transplantation therapy is likely to extend to PS cells.

Despite this unique immune status, numerous studies have shown that ES cells are still likely to express at least low levels of MHC proteins and ignite an immune response following allogeneic transplantation (reviewed by [36]). For example, primed T cells are able to recognize hES cells, and MHC-mismatched ES cells are rejected in immune competent mice [38]. ES cells with low MHC class I expression may also be rejected by natural killer (NK) cells [11, 39]. Therefore, significant efforts are currently underway to produce pluripotent stem cells that are MHC-matched to the recipient for transplantation therapies.

8.5.2 PS Cells to Overcome Histoincompatibility

PS cells represent a unique cell type for overcoming histoincompatibility of tissues during transplantation therapy. PS cells are a potential source of pluripotent cells personalized to the oocyte donor (and other individuals), expressing the same HLA

Table 8.1 Comparison of fertilized ES and PS cells

	Fertilized ES cells	Parthenogenetic ES cells
Source of blastocyst	Fertilized embryo	Artificially activated oocyte
Blastocyst capable of development to term?	Yes	No
In vitro differentiation (embryoid body, cell differentiation)	Pluripotent	Pluripotent
In vivo differentiation (teratomas, chimeras, TEC, transplantation)	Significant evidence for pluripotency in vivo and transplantation in animal models	Form teratomas, contribute to chimeras, very limited in vivo transplantation data
Maternally imprinted gene expression	Normal (some epigenetic errors reported)	Some overexpressed, some reprogrammed to normal levels
Paternally imprinted gene expression	Normal (some epigenetic errors reported)	Some not expressed, some reprogrammed to low or normal levels

proteins. A complete HLA/MHC match between the transplant tissue and the recipient is referred to as a histocompatible tissue source. Personalized HLA-matched PS cells may reduce or eliminate the need for immunosuppressive drug treatments, although this remains to be determined. Furthermore, the derivation of PS cells may be less ethically controversial than ES cells since parthenotes do not develop to term without significant genetic modification. Other methods for derivation of histocompatible pluripotent stem cells are limited by ethical controversy and technical limitations (nuclear transfer) or significant genetic manipulation (induced pluripotent stem (iPS) cells). The efficiency of PS cell derivation from human oocytes is reasonable and supports the notion that human PS cells may be routinely derived if oocytes are available [20].

PS cells hold promise as a source of personalized stem cells (to the oocyte donor) or as a resource for stem cell banking. Both MHC-homozygous and MHC-heterozygous human PS cells have been described, and each may have potential advantages as a tissue source [11, 20, 21] (summarized in Tables 8.1 and 8.2).

PS cell lines may be homozygous at the MHC under three different circumstances: (1) the oocyte donor is MHC-heterozygous, but recombination during oocyte activation does not include the MHC [11], (2) the oocyte donor is homozygous for each MHC allele (less likely) [21], and (3) the oocyte donor is heterozygous, but activation procedure results in a fully homozygous genome [18, 21] (Fig. 8.3d). Homozygosity at the MHC may produce a histocompatible cell source for a larger percentage of the population than heterozygosity at the MHC, since fewer alleles would need to be "matched" with the graft recipient.

This relates to the idea of creating stem cell banks with cell lines that are suitable for transplantation therapy. Taylor et al. [40] estimated that 150 ES cell lines (derived from fertilized embryos) would be sufficient to maintain a bank of ES cell lines that had at least a "good" MHC match to a large percentage of the British population. In contrast, only 10 MHC/HLA homozygous cell lines, such as

Table 8.2 Potential benefits and limitations of PS cells compared to fertilized ES cells

	ES cells	MHC-heterozygous PS cells	MHC-homozygous PS cells
Benefits	Simple derivation procedure	Does not create a fertilized embryo	Does not create a fertilized embryo
	May be used to create a stem cell bank (about 150 cell lines)	May be used to create a stem cell bank (about 150 cell lines)	Fewer cell lines (about 10) needed for stem cell bank
	Completely heterozygous genome	Histocompatible to oocyte donor	Histocompatible to a larger percentage of population
Limitations	Ethical controversy (use of fertilized embryo)	Some imprinting errors, less research on differentiation potential	Some imprinting errors, less research on differentiation potential
	Histocompatible to small percentage of the population	Histocompatibility limited to oocyte donor, relatives, and small percentage of population	MHC homozygosity may induce immune response in some transplantation types
		Genome is partially homozygous, which could harbor harmful recessive genes	Genome is partially homozygous or completely homozygous (depending on activation procedure), which could harbor harmful recessive genes

from MHC-homozygous PS cells, would be needed to maintain a stem cell bank with similar population matching. However, completely homozygous stem cell lines (Fig. 8.3d) may represent a clinical safety risk since any harmful recessive alleles could be expressed.

MHC/HLA heterozygous PS cells are produced when recombination during oocyte activation includes the genes of the MHC (Fig. 8.2b, c). These MHC-heterozygous PS cells and their differentiated tissues would be completely histocompatible to the oocyte donor and potentially histocompatible to close relatives of the oocyte donor. MHC-heterozygous (and matched) tissues may be advantageous in some types of transplantation to reduce possible natural killer (NK) cell rejection of cells with only one set of MHC genes, a phenomenon known as "hybrid resistance" noted in bone marrow transplants (reviewed in [41]). It is important to note that even MHC-heterozygous PS cells have regions of homozygosity within each chromosome (Fig. 8.3b, c). It remains to be determined whether MHC-homozygous or MHC-heterozygous PS cells are better suited for histocompatible transplantation therapy, although this may depend on the tissue or type of transplantation.

8.5.3 Current Research on Transplantation with PS Cells

Although the research to date on PS cells for transplantation therapy is limited, the results have been extremely promising. Both MHC-homozygous and MHC-heterozygous mouse PS cells formed teratomas in immune competent MHC-matched mice following subcutaneous injection [11]. MHC-heterozygous PS cells (C57BL/6 and CBA MHC background) only formed teratomas when injected into MHC-matched heterozygous mouse strains, while the cells were rejected when injected into MHC-homozygous (either C57BL/6 or CBA) mouse strains. Similarly, MHC-homozygous PS cells formed teratomas in either MHC-homozygous or MHC-heterozygous recipients [11]. Importantly, the mice in these experiments were immune competent and did not receive immunosuppressive therapy in order to accept the MHC-matched PS cell grafts. These experiments illustrate the potential of both MHC-homozygous and MHC-heterozygous PS cells to be used as a source of histocompatible cells for transplantation therapy.

Parthenogenic neurons have recently shown therapeutic success following transplantation in a monkey model of Parkinson's disease [42, 43]. Primate PS cells (Cyno-1) were differentiated to dopaminergic neurons in vitro using a protocol of initial co-culture with stromal cells followed by stepwise addition of specific signaling factors. Following transplantation into rat and primate midbrains, a small percentage of these PS cell-derived neurons survived for months and did not form teratomas [42]. Further studies showed that dopaminergic neurons survived without teratoma formation and improved motor skills in primates in a Parkinson's disease model [43]. This represents the first demonstration of cellular function following parthenogenic tissue transplant.

8.6 Conclusions

The efficient derivation of pluripotent PS cells from multiple species highlights their potential for generating tissues for transplantation therapy. While parthenotes are not fertilized by sperm and cannot develop to term, PS cells are pluripotent and may contribute to all cell types of the body, similar to ES cells. While some errors in imprinting remain in PS cells, the degree to which these cells may be reprogrammed and the extent to which imprinted genes affect the therapeutic potential of PS cells remains to be seen. MHC-homozygous and MHC-heterozygous PS cells may be derived and each has potential advantages for overcoming histoincompatibility of stem cell transplants. Therefore, PS cells may be a source of histocompatible tissues for the oocyte donor, her relatives and potentially to a large proportion of the public through stem cell banking. The use of histocompatible PS tissues may reduce or eliminate the need for immunosuppressive drugs following transplantation. If federal funding restrictions are relieved, increased research and publications on the safety and efficacy of parthenogenic tissues will inevitably follow.

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Chapter 9
Prospects for Designing 'Universal' Stem Cell Lines

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Abstract Successful transplantation of conventional tissues between individuals requires matching of human leukocyte associated antigens (HLA), in order to prevent rejection. Although the same principles apply to tissues differentiated from embryonic stem (ES) cells, recent advances in gene delivery and genetic regulation have raised the prospect of engineering grafts with reduced levels of HLA expression. This strategy may mitigate the effects of extensive HLA polymorphism which restricts the availability of suitable donors and necessitates the maintenance of large donor registries. Here, we discuss the potential of employing RNA interference (RNAi) to knockdown HLA expression, enabling allogeneic cells to evade immune recognition. We discuss how lentivirus-mediated delivery of short hairpin RNAs (shRNA) targeting pan-class I and allele-specific HLA achieves efficient, dose-dependent reduction in surface HLA expression in human cells. Thus, by combining genetic engineering and regenerative medicine, RNAi-induced silencing of HLA expression has the potential to create histocompatibility-enhanced and, perhaps even, "universally" compatible cellular grafts.

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9.1 Introduction

9.1.1 Histocompatibility Antigens, the Major Cause of Allorecognition and Graft Rejection

Immune responses against donor (i.e., "non-self" or "allogeneic") antigens are the primary cause of rejection of transplanted cells and tissues, resulting in graft failure. The major histocompatibility complex (MHC) in humans, also known as the human leukocyte associated antigen (HLA) system, plays a critical role in immunological discrimination between "self" and "non-self". The HLA system consists of a family of polymorphic genes situated on chromosome 6, which encode cell surface proteins that present antigenic peptide sequences to T cells (Fig. 9.1). Of the several genes encoding HLA antigens, the most important for allograft survival have been considered to be class I antigens HLA-A and -B, and class II antigen HLA-DR.

HLA genes are among the most highly polymorphic in the human genome, and with the advent of high-throughput DNA sequencing, there are an ever-increasing number of allelic variants that are being identified. More than 2,500 new HLA alleles have been identified since 2005, and at present, more than 965, 1,543, and 858 epitopes have been molecularly defined for A, B, and DR loci, respectively [1].

HLA class I antigens are expressed on virtually all cell types except in the central nervous system, and mediate the presentation of a complexed self or non-self peptide epitope for T-cell recognition. Polymorphisms of HLA class I alleles generally result in structural variations of the antigenic peptide binding groove, which in turn affects its peptide binding affinity and therefore the repertoire of antigenic epitopes that can be presented. These polymorphic structural variations also represent the basis for discrimination of "self" versus "non-self", and presentation of a foreign "non-self" peptide to $CD8^+$ T effector cells can activate their cytotoxic function. Also of importance, class I antigens appear to act as inhibitory ligands that prevent cytolytic attack by natural killer (NK) cells.

In contrast, class II antigen expression is generally restricted to B cells, activated T cells and antigen-presenting cells (APC) such as macrophages and dendritic cells. Notably, however, class II expression can be induced on other cell types by interferon (IFN)-γ, and is up-regulated in inflammation. Class II antigens also present peptide epitopes, primarily to $CD4^+$ T helper cells, and accordingly, polymorphisms of class II alleles also affect the repertoire of antigenic peptides that can be presented to $CD4^+$ T cells and thereby become targets for elimination by B cell-mediated humoral responses.

Allogeneic HLA can be recognized by two major mechanisms. One mechanism involves direct recognition by "self" $CD8^+$ and $CD4^+$ T cells of intact allogeneic HLA class I or class II on donor APC as "non-self" antigens (Fig. 9.2a). Alternatively, allogeneic donor HLA may be processed into antigenic peptides by recipient APC for presentation in the context of "self" HLA class II to $CD4^+$ T cells, or for cross-presentation to $CD8^+$ T cells in the context of "self" HLA class I, resulting in

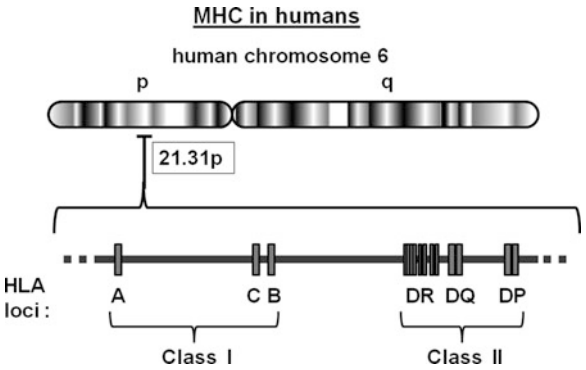

Fig. 9.1 The human major histocompatibility complex (MHC). Human chromosome 6, which contains the MHC residing at 21.31p, is depicted at the top. There are six genetic loci that encode HLA antigens associated with rejection versus survival. Note the close association (linkage) between DR-DQ and B–C

"indirect" recognition of "non-self" antigens (Fig. 9.2b). Indirect recognition is, therefore, limited to "self"-MHC-restricted epitope targets derived from "non-self" HLA, but direct recognition is not MHC-restricted, and therefore can greatly increase the number of epitopes targeted by cellular and humoral allogeneic responses.

9.1.2 Barriers to Conventional Organ and Tissue Transplantation

To date, the primary strategies for avoiding immune rejection of transplanted cells and organs have been to minimize antigenic differences between donor and recipient by matching HLA alleles, and to administer potent immunosuppressive drugs to the transplant recipient.

Mismatching of serological antigens is enough to increase the probability of graft failure [2–4], and even when serology is matched, small molecular genetic differences may cause transplant rejection [5–7]. Matching HLA -A, -B, and -DR alleles has been found to have a significant effect on graft survival versus rejection in organ transplantation of kidneys, as well as pancreas, heart, lung, and bone marrow transplantation [8]. While the influence of matching on outcome of liver graft survival has been controversial, and matching HLA-DR has no beneficial effect, it appears that matching HLA -A and -B alleles may be significantly associated with lower rates of graft rejection. Furthermore, particularly in the case of bone marrow and hematopoietic stem cell transplantation, recipient HLA antigen mismatching to the donor can cause graft versus host disease (GvHD), which may have severe complications [9, 10].

Since HLA alleles are highly polymorphic, the better the match between donor and recipient, the more limited will be the supply of compatible donors. Indeed, there is currently an overwhelming shortage of donors compared to the number of potential recipients, who must remain on a waiting list until a suitable HLA-compatible donor is found. All too frequently, the patient requiring a transplant succumbs to organ failure or to the underlying disease before a matching donor can

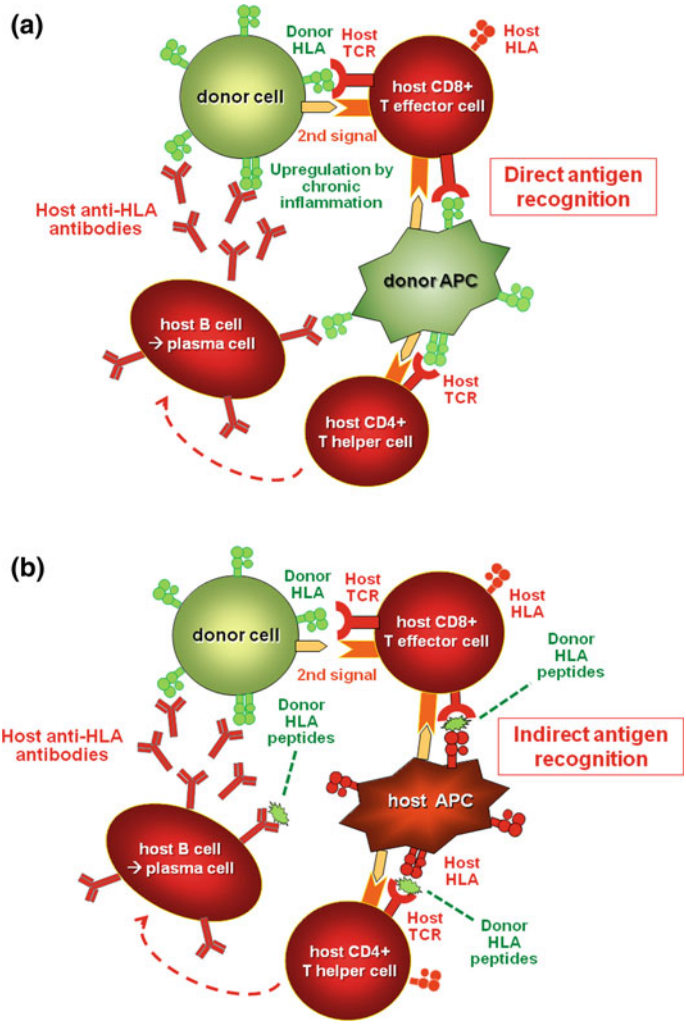

Fig. 9.2 Allograft rejection: immunological mechanisms and strategies for evasion. Two potential mechanisms for allograft rejection are depicted attacking the graft cell, resulting in its elimination: **a** Direct antigen recognition, in which T-cell receptors (TCR) on host T cells recognize intact donor HLA molecules on the graft cells as non-self, presumably because their three-dimensional structure resembles a self MHC bound to a foreign peptide ("molecular mimicry"). **b** Indirect antigen presentation, in which peptides derived from donor HLA molecules are presented by host APC as foreign antigens. Either mechanism can induce both CTL-mediated cellular responses, as well as humoral responses involving host antibodies against donor HLA that bind and initiate graft damage through antibody-dependent cellular toxicity and complement activation. Note that HLA class II and co-stimulatory molecules such as CD80 and CD86 can also be up-regulated in donor non-APC cells upon inflammation. Co-stimulatory molecules provide a critical "2nd signal" that activates T cells upon HLA engagement by T cell receptor (TCR), while HLA class II can also become a target for immune rejection

be found. Even when a suitable donor can be identified, the need to transport harvested organs over large distances to the recipient while maintaining tissue viability presents further technical and logistical difficulties. Thus, the extensive polymorphism of HLA alleles and the shortage of suitable HLA-matched donors represent the foremost limitations to the field of organ transplantation.

With the advent of contemporary immunosuppressive drugs, it had been hoped that the importance of HLA matching might be reduced [11, 12]. Indeed, most reports have shown a smaller HLA effect in the short term, as acute cellular rejection can now usually be readily reversed by immunosuppressive therapy with high-dose corticosteroids and calcineurin inhibitors. However, chronic humoral rejection is frequently refractory to such treatments, and recent data suggest that matching of donor and recipient HLA alleles provides an additive increase in long-term graft survival when combined with calcineurin inhibitors [8, 11–13]. Furthermore, the adverse consequences of long-term administration of immunosuppressive drugs, including infection and toxicity to the recipient, and in some cases post-transplant malignancies, are well recognized.

9.2 Pluripotent Stem Cell-Derived Transplants: Still Subject to Immune Rejection

The same immunological obstacles that have long confronted the field of adult organ and tissue transplantation also represent one of the most important challenges to clinical application of human embryonic stem (hES) cell derived cells and tissues. As hES cells do not express HLA class II and only barely detectable HLA class I in their native undifferentiated state, initially it was suggested that some degree of immune privilege might be conferred by unique properties that inhibit maternal immune responses to the fetus [14]. However, it is now recognized that expression of endogenous HLA class I is highly up-regulated when hES cells differentiate into various lineages [15]. Presumably, this will render non-autologous hES cell-derived mature differentiated cells susceptible to immune rejection, upon transplantation into a non-HLA-matched recipient host.

In fact, the immunological consequences in vivo remain unclear, with conflicting reports suggesting that hES cell-derived transplants are tolerated in immunodeficient mice reconstituted with human immunocytes [16], while in other cases are rejected by adaptive or innate immunity [17]; some of these differences may be due to heterogeneity of hematopoietic cells derived from hES cells, which may include tolerogenic APCs and immunosuppressive Treg cells. Improving engraftment by generation of donor hES cell-derived tolerogenic cells is also being pursued [18–20], but these approaches tend to be handicapped by our incomplete understanding of the complexity of immunoregulatory mechanisms, which again, present the same obstacles that still have not been overcome, even in the field of adult organ and tissue transplantation.

More recently, it has been discovered that the combined expression of the transcription factors Kruppel-like Factor 4 (KLF4), POU5F1 (OCT3/4), SOX2, and c-MYC can achieve reprograming of differentiated cells from mice and humans into induced pluripotent stem (iPS) cells [21–24]. Of course, under most circumstances, autologous iPS cell-derived cells and tissues should not be subject to immunological rejection, unless co-expression of foreign proteins derived from virus or plasmid vectors employed for gene delivery of reprograming factors are recognized by the immune system. However, in the case of hereditary disease processes, cells and tissues derived from autologous iPS cells would still retain the original genetic defect. In such cases, the autologous iPS cell-derived cells would first need to undergo corrective gene transfer or other measures to restore normal function before they could be used as a source of regenerative tissue, and the development of such individualized cellular therapies would entail custom cGMP manufacturing processes for ex vivo genetic correction. Furthermore, while tissue-specific and homeostatically regulated transgene expression is not always required for correction of hereditary genetic defects, in some cases (e.g., insulin gene expression), the lack of such controlled expression from exogenously introduced transgenes could prove deleterious to the patient. Yet, all too often, the transcriptional and post-transcriptional regulatory sequences needed for fully normal expression of specific genes are not known, and in some cases, critical regulatory elements may reside in genetic locus control regions that are hundreds of kilobases upstream or downstream, or within introns. Hence, based on previous experience in the field of gene therapy, it is probable that achieving normal levels and appropriate regulation of corrective transgene expression in specific tissues will, in many cases, prove challenging.

As an alternative, in such cases it is likely that normal hES cell- or iPS cell-derived cells and tissues from an allogeneic source will still need to be used, and in fact this may represent a preferable solution, as these cells would naturally express a normal version of the patient's defective gene in its normal genomic/chromatin context, i.e., with all genetic, epigenetic, and cellular regulatory mechanisms needed for normal expression maintained intact. Additionally, the use of genetically normal hES cell- or iPS cell-derived cells and tissues derived from a well-characterized Master Cell Bank would represent a more generalized solution that may avoid many of the problems associated with custom manufacture of individualized autologous cell therapies. Again, however, in this case, the origin of such "off-the-shelf" hES cell- or iPS cell-derived regenerative therapies will necessarily be allogeneic in nature.

9.3 Reducing Histocompatibility Barriers by Conditioning the Graft, Not the Host

Recent advances in techniques for gene delivery and cell engineering now make it possible to envisage novel strategies to genetically modify the graft cells in order to evade or inhibit immunological rejection. This represents a paradigm shift compared to traditional immunomodulatory strategies in transplantation, which

generally seek to make the host more accepting to the engrafted tissue by systemic administration of potent immunosuppressive drugs.

We and others have, therefore, been exploring strategies for engineering cells with post-natal genetic modifications that reduce or even eliminate HLA expression, either globally or in an allele-specific manner. In principle, such modifications could allow grafts to evade alloreactive immune responses and thereby reduce or eliminate the need for HLA matching, as well as the need for lifelong immunosuppression of the host (Fig. 9.3a), and thereby help to overcome the histocompatibility barriers to conventional organ and tissue transplantation imposed by HLA polymorphism. By the same token, this novel strategy could prove useful in overcoming HLA incompatibilities that also threaten to be a major barrier to allogeneic stem cell-based regenerative therapies [25, 26].

If targeted to critical molecules involved in immunorecognition or effector function, the net effect of such manipulations should be to decrease immunogenicity of donor hES cell-derived transplants and reduce the recipient immune response. While complete elimination of HLA will render donor cells susceptible to recognition and attack by non-HLA-restricted effector cells, such as natural killer (NK) and lymphokine-activated killer (LAK) cells, it may be possible to modulate the level of HLA expression to a window that is insufficient for recognition by alloreactive T cells and yet will not attract NK cells.

Alternatively, precise targeting of specific HLA alleles may be used to nullify individual mismatches. As there are only a limited number of hES cell lines currently available, the majority of potential recipients of hES cell-derived grafts would likely exhibit mismatched HLA alleles compared to the available hES cell donor cells. Hence, the ability to nullify specific mismatched HLA alleles would greatly increase the histocompatibility and utility of existing hES cell donor cells, while maintaining resistance to NK cell attack.

9.4 HLA Down-Regulation as an Effective Immune Evasion Strategy

Various strategies previously tested to condition the graft for immune evasion, such as pre-treatment with antibodies or immunoconjugates directed against graft haplotypes, APC, adhesion molecules, or costimulatory factors, proved largely ineffective in promoting acceptance of allografts in immunocompetent hosts [27]. However, it has been demonstrated that allografts from MHC class-I- and class-II-knockout mice could survive significantly longer than controls [27, 28], suggesting that immune evasive strategies might be successful, if more efficient and/or long-lasting HLA inhibition could be achieved. In fact, Wayne Marasco and Martina Seifert et al. subsequently showed that gene transfer of an anti-human MHC class I single-chain intracellular antibody (intrabody) could achieve 'phenotypic knockout' of HLA class I in primary human keratinocytes and endothelial cells [29, 30]. Significantly, it was reported that intrabody-transduced cells were resistant to lysis

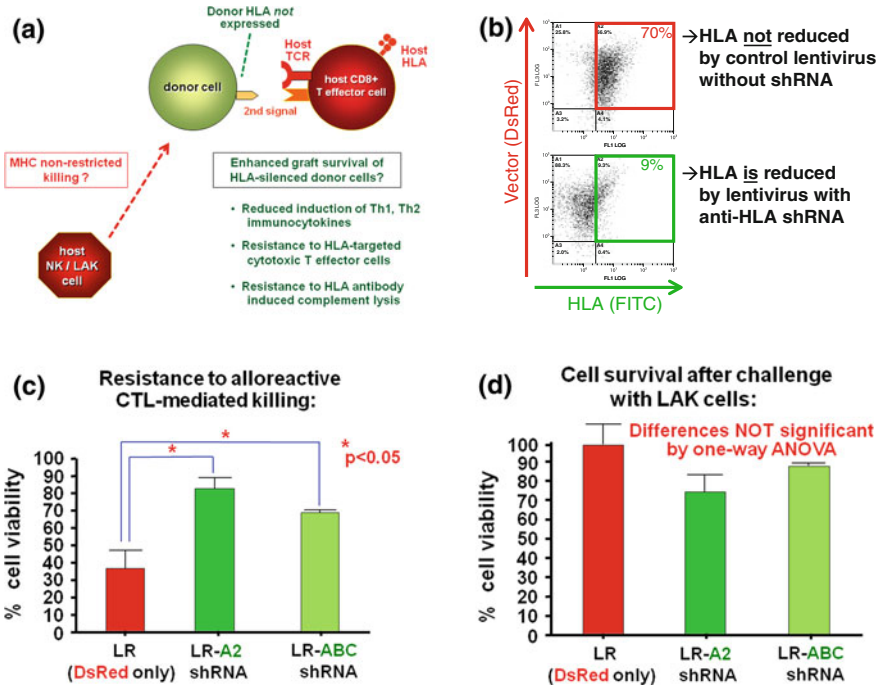

Fig. 9.3 Lentiviral vector-mediated shRNA knockdown of HLA expression. **a** This schematic depicts the concept of immune evasion by engineering donor cells to reduce their HLA expression: although co-stimulatory molecules such as CD80 (2nd signal) might be displayed, none of the direct or indirect recognition mechanisms would be activated in the absence of HLA on the donor cells. This could lead to prolonged graft survival by making the donor cells 'invisible' to alloreactive immune responses, but could also incur MHC-non-restricted killing by host NK or LAK cells. **b** Human embryonic kidney (293T) cells were transduced with either control lentiviral vector expressing the DsRed marker gene only (LR vector), or lentiviral vectors expressing the pan-class I HLA-ABC-specific shRNA in addition to DsRed (LR-ABC vector) or HLA-A2 allele-specific shRNA in addition to DsRed (LR-A2 vector). Cell surface HLA expression was detected by FACS analysis using a fluorescein isothiocyanate (FITC)-conjugated anti-HLA-ABC (FITC-anti HLA-ABC Ab) antibody. Control LR vector-infected cells stained with FITC-conjugated HLA antibodies show a shift in both DsRed fluorescence (Vector; Y axis) and FITC fluorescence (HLA; X-axis) to the upper right quadrant (red box). In contrast, with LR-ABC vector transduction, in the presence of FITC antibodies there is only a shift up the DsRed axis and very little shift along the FITC axis, indicating that there is nothing for the anti-HLA antibodies to bind to, and demonstrating that successful knockdown has been achieved in a dose-dependent manner. **c** 293T target cells transduced with control lentivirus vector expressing DsRed only (LR) continue to express HLA-A2/B7/Cw7, and show <40 % viability by MTS assay 48 h after incubation with alloreactive CTL that had been activated against stimulator cells expressing HLA-A2/B44/C5. In contrast, significantly increased viability, indicating resistance to alloreactive cytotoxicity, is observed in target cells transduced with pan-specific (LR-ABC) or allele-specific (LR-A2) vectors ($p < 0.05$ for both compared to control, as indicated). Incubations were performed at an alloCTL (effector) : 293T (target) cell ratio of 10:1. Results are expressed as mean values ± SEM in % viability. **d** HLA knockdown does not increase non-MHC-restricted killing by LAK (non-MHC-restricted effector) cells, as cell viability measured by MTS assay was not significantly reduced 48 h after incubation at a LAK (effector): 293T (target) cell ratio of 10:1. Results are expressed as mean values ± SEM in % viability, and were analyzed by one-way ANOVA

by alloreactive cytotoxic T lymphocytes (CTL), while control cells from the same donor remained unprotected [30].

These studies were, thus, noteworthy in establishing proof-of-concept for immunogenicity reduction of adult tissue allografts by targeted inhibition of HLA. In this context, however, it should be noted that the choice of gene transfer technology used for genetic modification ("transduction", in the parlance of the field) of the intended target cells is quite an important consideration, particularly when contemplating how to achieve long-term, possibly lifelong, down-regulation of HLA expression. For example, in the case of the gene transfer study mentioned above, an adenovirus-based gene delivery vehicle ("vector") was used to deliver the intrabody coding sequence for intracellular expression. However, the natural life cycle of wild type adenoviruses normally ends in cytolysis of the host cell, and accordingly, vectors derived from this type of virus have no mechanisms to integrate stably into the host cell chromosomes except by chance, a rather rare occurrence (generally on the order of 1 in 100,000–1,000,000 vector-transduced cells). Consequently, adenovirus vector DNA in the transduced cell exists largely in the form of extrachromosomal episomes, which are progressively lost over time as turnover occurs due to host cell mitosis, resulting in limited duration of transgene expression. Furthermore, it is now well established that conventional adenovirus vectors elicit robust cellular and humoral anti-viral immune responses in vivo, which result in elimination of vector-transduced cells over time.

Of note, a number of groups have pursued the development of helper-dependent (aka "gutless") adenovirus vectors, from which all viral coding sequences have been fully eliminated, resulting in a significantly reduced cellular immune responses against the vector and longer duration of transgene expression in vivo. The same appears to hold true for vectors derived from adeno-associated viruses (AAV), which are much smaller in genome size, have a much more limited packaging capacity, and which are now also known to form concatameric extrachromosomal episomes. Thus, in either case, transgene expression levels from the vector episomes will decline over time at a rate proportionate to the level of cellular turnover. Moreover, the synthetic anti-MHC intrabody protein itself also represents a foreign antigen, which may become a target for immune attack as vector expression wanes. Hence, it may prove difficult to achieve long-term HLA down-regulation and concomitant graft survival with this approach. In this regard, RNA interference represents a newer technology that does not require expression of any foreign proteins, and has the potential to achieve efficient and highly selective silencing of targeted genes.

9.5 Application of RNAi -Based Methods for Knockdown of HLA Expression

RNA interference (RNAi) has emerged as an important gene regulatory mechanism that affects sequence-specific knockdown of mRNAs [31], and which can also be employed as a potent genetic tool for silencing gene expression [32–34].

Short double-stranded RNAs, called small interfering RNAs (siRNAs), can be used to modulate gene expression by triggering post-transcriptional degradation of complementary messenger RNA transcripts through a multistep mechanism. A protein complex called the RNA-induced silencing complex (RISC) incorporates one of the siRNA strands and uses this strand as a guide to recognize target mRNAs. Depending on the complementary interaction between guide RNA and mRNA, RISC then destroys or inhibits translation of the mRNA. A perfect complementary match results in mRNA cleavage and degradation, whereas a partial complementary match (particularly with sites in the 3' untranslated region) results in translational inhibition. RNAi is conserved in most eukaryotes and can, by introducing exogenous siRNAs, be used as a tool to down-regulate specific genes. Furthermore, it has recently been recognized that, although RISC primarily acts at the post-transcriptional level, RNAi can also mediate transcriptional gene silencing in a variety of organisms, perhaps through small non-coding RNAs that act through homologs of the RISC complex, which apparently recruit histone-modifying proteins to regulate chromatin structure at their genomic loci [35–37].

John Rossi, Laurence Cooper, and colleagues [38] first demonstrated that siRNA could achieve HLA down-regulation in T cells, providing protection from cytolysis even when the transfected cells were loaded with a target peptide and challenged with peptide-specific CTLs. However, the procedure entailed chemical transfection of plasmids into an immortalized Jurkat T cell line for high copy number expression of siRNA, after which stable transformants were isolated and selected using an antibiotic resistance marker. This methodology would not be feasible for many cell types, including quiescent tissue-specific stem cells as well as post-mitotic differentiated somatic cells. It is possible that ES and iPS cells could be selected in this manner, but chemical transfection efficiencies are generally quite low, and run the risk of unwanted cellular differentiation or loss of cell viability during the selection process.

Again, the advantages and disadvantages of different gene transfer technologies should be taken into consideration. Based on lessons learned from the field of gene therapy over the past two decades, we and others have been developing lentivirus-based vectors for short-hairpin (shRNA)-mediated knockdown of HLA expression. Lentivirus-based vectors, which are capable of highly efficient transduction of quiescent cells including stem cells, and which readily achieve permanent integration into the host cell genome, provide the potential to develop a clinically feasible approach for long-term suppression of HLA.

9.6 Lentivirus-Based Vectors for Efficient and Stable Genetic Modification of Stem Cells

The past 15 years have witnessed the progressive development of highly efficient gene delivery vector systems derived from different lentiviruses, including human immunodeficiency virus (HIV), simian immunodeficiency virus (SIV), feline

immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV), some of which are now being tested in clinical trials. Lentiviruses are complex retroviruses that contain "accessory" genes encoding regulatory (*tat, rev*) and pathogenicity-enhancing (*vif, vpr, vpu, nef*) functions, in addition to the *gag*, *pol* and *env* structural proteins, classically expressed by simple oncoretroviruses.

Unlike adenovirus and AAV vectors, the hallmark of the lentiviral life cycle is their use of reverse transcriptase to convert the viral RNA genome to double-stranded DNA, which is then permanently integrated into the chromosomes of the host cell. Of course, this is also a characteristic of classic retroviral vectors based on simple oncoretroviruses such as Moloney murine leukemia virus (MLV). However, a notable difference is that oncoretroviral vectors can only transduce cells that divide shortly after infection, because entry of the MLV pre-integration complex into the nucleus is completely dependent on dissolution of the nuclear envelope which occurs during mitosis. In contrast, lentiviruses such as HIV can infect non-proliferating quiescent cells, owing to the presence of nuclear localization signal (NLS) sequences in their pre-integration complex, which allow recognition and active uptake by the cell's nuclear import machinery.

So-called "third-generation" lentiviral vectors and their packaging systems [39, 40], which represent the current "industry standard", are multiply attenuated by deletion of the accessory genes *vif, vpr, vpu, nef* and *tat*. The only auxiliary gene remaining in the packaging system is therefore *rev*, which, along with the Rev response element (RRE) as its cognate *cis*-binding sequence in the vector, is required for efficient export of the vector and packaging construct RNAs from the nucleus during virus production. Thus, both toxicity as well as the likelihood of recombination are reduced in third-generation lentiviral vector systems, which are now commercially available.

Lentiviral vectors are generally pseudotyped (i.e., coated with a heterologous envelope protein) with vesicular stomatitis virus glycoprotein (VSV-G) to achieve wider host range and stability of virions. To generate virus, the vector construct is generally transiently co-transfected along with a *gag-pol* packaging construct, the *rev* accessory gene construct and VSV-G *env* construct into 293T cells to produce virus [39]. Assembly of these viral proteins is initiated by a *cis*-acting sequence located next to the 5' LTR in the vector, known as the packaging signal (Ψ), which enables the vector mRNA to be packaged into virions. Such transient transfection systems achieve high level expression of viral proteins and efficient packaging of vector genomes without the need for long-term maintenance of stable packaging cell lines, and thus without the attendant risk of recombination leading to generation of helper virus over time.

Another advantage of lentiviral vector systems is that the endogenous promoter in the HIV long terminal repeat (LTR) depends on the HIV-encoded Tat transctivator protein for transcriptional function. As the sequences encoding Tat are removed from lentiviral vectors, there is little promoter activity from the LTR, and effective transgene expression is dependent on the addition of an internal promoter. Of note, it has previously been found that, despite the lack of significant promoter activity in the absence of Tat, interference between the HIV LTR and the internal promoter can occur, significantly attenuating the levels of transgene expression achieved.

Fortunately, this has been largely overcome by the use of self-inactivating (SIN) vectors, in which a portion of the U3 region of the 3' LTR has been deleted [40]; thus, after reverse transcription, this deletion is copied to the 5' LTR and hence results in loss of LTR promoter sequences in the integrated provirus, which therefore prevents interference with the function of the internal promoter. Although many lentiviral constructs contain internal CMV promoters to drive transgene expression, the use of tissue-specific or conditional (e.g., tetracycline-responsive) internal promoters may be highly advantageous when more precise control of expression is required. Internal promoters may also be important for long-term gene expression, as silencing of CMV promoter-driven transgene expression over time in some lines of cells is avoided or eliminated. Furthermore, Pol III promoters such as U6 or H1 are required for direct expression of shRNA, although newer intron-based miRNA designs can be driven by regular Pol II promoters (see below).

Accordingly, we have focused primarily on these third-generation SIN lentiviral vectors for genetic engineering of cells to efficiently effect long-term stable changes in cellular function and phenotype. We and others have confirmed that lentiviral vectors can transduce cell lines that are growth-arrested in culture, as well as terminally differentiated primary cells, including hematopoietic stem cells, neurons, hepatocytes, cardiomyocytes, endothelium, alveolar pneumocytes, keratinocytes, and dendritic cells [41–49]. As noted, once nuclear entry by the lentiviral vector has occurred, the reverse-transcribed vector sequence is permanently integrated into the host cell genome, enabling stable long-term expression of transgenes or shRNA.

9.7 Identification of Target Sequences for Knockdown of HLA Expression

Once an optimal system has been established for shRNA delivery to achieve long-term stable down-regulation of HLA antigens, the question then arises as to which specific sequences should be targeted. From the above information regarding the relative importance of different HLA antigen loci in transplant graft rejection, the preferred HLA antigen targets would be the class I antigens HLA-A and -B, and the class II antigen HLA-DR. Any exon comprising each of these antigens may contain sequences that are useful targets for shRNA- or antisense RNA-mediated inhibition. Further, these target proteins are not necessarily the only targets available, and other targets also include, but are not limited to, immunostimulatory co-activators such as CD80 (B7.1), CD86 (B7.2), etc., which may also be expressed on the surface of certain donor graft cells.

As noted, HLA antigens are highly polymorphic; indeed, this is the very reason for the need to match HLA types between donor and recipient. However, certain regions within the HLA sequence are highly conserved and non-polymorphic; if targeted, these regions allow global inhibition of HLA expression in the donor cells regardless of the specific HLA type of the donor. HLA class I antigens share the same general structure, being composed of a heavy chain consisting of three

alpha (α) domains, which are non-covalently paired with a smaller chain known as beta-2 (β2)-microglobulin. The heavy chain and β2-microglobulin associate with each other, along with antigenic peptides, in the endoplasmic reticulum, and are transported together to the cell surface. In particular, the α3 domain has a non-polymorphic loop that interacts with CD8, and the β2-microglobulin subunit is invariant. These conserved sequences are the preferred targets for general inhibition of HLA class I expression.

For down-regulation of a specific HLA antigen to nullify a mismatch at a particular HLA locus in an otherwise well-matched donor graft, the highly polymorphic sequences in the α1 domain or α2 domain of the HLA class I antigens can be targeted. Based on known sequence information for individual HLA antigens, one can readily identify the appropriate target sequences for inhibition of a specific HLA allele. Thus, for example, if a donor graft is well matched at both alleles in the HLA-B and -DR loci, and is matched at one allele in the HLA-A locus but is mismatched at the other (i.e., a 5 out of 6 match), the α1 and α2 domain sequences of the specific HLA-A allele that is mismatched in the donor can be determined from a search of available HLA sequences, and these unique sequences would be used to target only this particular antigen for suppression in the donor graft cells, thereby nullifying the mismatch and essentially converting the graft into a "5 out of 5" perfect match which would significantly enhance graft survival.

It should be noted that a suitable stretch of coding mRNA for targeting by siRNA or antisense RNA has the following properties: secondary structures do not appear that would hinder silencing; there must be a minimum of non-identity, as a single nucleotide that is non-complementary might abolish silencing; the cDNA nucleotide sequence must begin with a guanine since that base is the start site of the U6 promoter. Guidelines of properties for efficient siRNA further include: length of 21–23 nucleotides as a length of double-stranded RNA greater than 30 triggers interferon response and cell death; the G + C content must be about 50 %; and cDNA sequences preferably conform to the following, in order of preference: AA(N19)TT > NA(N21) > NAR(N17)YNN, where N, Y, and R are the conventional symbols for any nucleotide, a pyrimidine, and a purine, respectively.

9.8 Development of Lentiviral Vectors for Knockdown of HLA Class I Expression

9.8.1 HLA-Targeted shRNA Vector Design

Generally, it may be necessary to screen at least four or five siRNA candidate sequences to identify one that exhibits highly potent knockdown activity. Accordingly, we performed a sequence alignment to identify several polymorphic sequences in the α1 and α2 domains that are divergent between the most frequently represented HLA-A, -B, and -C alleles in the Caucasian population, to design an

allele-specific siRNA specifically targeting HLA-A0201. We also identified highly conserved and non-polymorphic sequences in the class I $\alpha 3$ domain and the invariant $\beta 2$-microglobulin subunit to be targeted by *pan-specific siRNAs*. As per the strategy outlined above, several HLA-A0201 allele-specific and HLA-ABC pan-specific siRNA sequences were designed as short hairpin RNA loop structures and inserted into HIV-derived lentiviral vector pLentiLox-DsRed. This vector contains a U6 promoter suitable for driving expression of shRNA constructs, as well as a separate DsRed fluorescent protein marker gene cassette flanked by loxP sequences, which allow its removal with Cre recombinase.

We first screened these HLA-targeted lentiviral shRNA vectors in 293T human embryonic kidney cells, an established cell line that predominantly expresses HLA-A2, as well as HLA-B7 and -Cw7 at lower levels. Of the candidate sequences tested, the highest silencing activities were obtained with an allele-specific shRNA construct targeting the unique HLA-A0201 sequence 5′-GGATTACATCGCCCTGAAAG-3′, and a pan-specific HLA-ABC shRNA construct targeting the conserved HLA class I sequence 5′-GCTACTACAACCAGAGCGAG-3′. Transduction with these vectors at increasing multiplicities of infection (MOI; i.e., virus/cell ratio) resulted in a dose-dependent reduction in HLA levels. At higher MOIs, allele-specific and pan-specific shRNA vectors reduced cell surface expression of HLA-A by 50 % and overall HLA class I by >80 %, respectively, as compared to cells transduced with negative control lentivirus expressing only the marker gene (Fig. 9.3b) [50, 51].

9.8.2 Lentiviral shRNA Vector-Mediated HLA Class I Knockdown Confers Reduced Immunogenicity

We investigated the functional consequences of knocking down HLA class I expression in this model system, specifically to determine whether HLA knockdown would be associated with reduced immunogenicity in vitro [50, 51]. Alloreactive human CTL were pre-activated with stimulator cells expressing HLA-A2, and incubated with shRNA-transduced 293T cells at a ratio of 10:1 (effector/target cell ratio). Alloimmunogenicity was examined both by a surrogate assay measuring the level of IFN-γ production from alloreactive T cells by ELISA, as well as by direct measurement of 293T target cell apoptosis by annexin V (which binds to phosphatidylserine that is externalized on the outer membrane outer leaflet during early apoptosis), and by direct measurement of target cell viability by MTS assay (a chromogenic assay measuring redox activity in viable cells). The results showed that, compared to cells transduced with control vector expressing DsRed only, target cells transduced with HLA class I pan-specific shRNA and HLA-A0201 allele-specific shRNA vectors both induced significantly less IFN production from alloreactive T cells, and exhibited significantly enhanced resistance to T-cell-mediated killing in both annexin V and MTS assays ($p < 0.05$) (Fig. 9.3c).

Reduced HLA expression in allogeneic cells might also incur increased sensitivity to HLA non-restricted natural killer (NK) cell- and lymphokine-activated killer (LAK) cell-mediated cytotoxicity, per the "missing self" hypothesis [52]. However, after incubation with LAK cells generated from the same donor as the alloreactive T cells, we observed no statistically significant differences in survival of HLA class I pan-specific or allele-specific shRNA-transduced target cells, as compared to DsRed-only vector-transduced controls (Fig. 9.3d).

Ranier Blasczyk, Axel Seltsam, Constança Figueiredo and colleagues in Hannover, Germany have also reported that both constitutive and tet-inducible lentiviral vectors expressing shRNA cassettes targeting HLA-A and β2-microglobulin could achieve similar protection in HeLa cells, immortalized B cell lines, and peripheral blood monocytes [53, 54]. Notably, as in our studies, HLA expression was significantly reduced by 80–90 %, but was not completely eliminated. Figueiredo et al. confirmed that the transduced cells showed resistance to complement-dependent cytotoxicity by HLA-A-specific antibodies, and reduced alloreactive $CD8^+$ T cell proliferation and IFN-γ secretion, but also did not elicit NK cell reactivity [53, 54]. These findings again suggest that a 'therapeutic window' may exist for knocking down HLA sufficiently to confer resistance to T-cell-mediated killing, yet leaving enough residual HLA expression to avoid NK cell-mediated killing.

9.8.3 Lentiviral Vector-Mediated HLA Knockdown in Primary Human Stem Cells

This highly promising strategy is now being applied to different types of primary human stem cells, both of embryonic and adult origin. For example, we have transduced primary human $CD34^+$ hematopoietic stem cells with lentiviral vectors expressing HLA-targeted shRNA, and have confirmed efficient transduction as well as effective down-regulation of cell surface HLA expression. Depending on the MOI used, the level of overall transduction and concomitant HLA knockdown could be modulated, ranging from 10 to over 80 % reduction. Functional studies confirmed that such HLA knockdown can confer protection to transduced primary human $CD34^+$ cells when challenged in vitro with anti-HLA antibodies in complement-dependent cytotoxicity (CDC) reactions, a gold-standard clinical lab test for monitoring allograft rejection in transplant patients. As expected, primary human $CD34^+$ cells transduced with control vector expressing only the DsRed marker gene showed no significant differences in their sensitivity to HLA antibody-directed complement lysis, regardless of the level of lentiviral gene transduction. In contrast, transduction with HLA-targeted shRNA vectors was associated with progressive reductions in HLA antibody-directed complement lysis, with the overall level of resistance to cytolysis showing positive correlation with the level of vector transduction (Fig. 9.4a). Notably, at transduction levels of 80 % or more, the shRNA-transduced primary human $CD34^+$ stem cells showed

complete resistance to anti-HLA antibody-directed complement lysis (Lemp, N.A. et al., manuscript in preparation).

In recent work, Figueiredo et al. [55] have also deployed this technology to make HLA-null "universal" blood platelets after in vitro differentiation of megakaryocytes derived from transduced $CD34^+$ hematopoietic stem cells. If cGMP scale-up manufacturing of such HLA-null platelets can be achieved, this could have significant implications for transfusion medicine, as human platelet preparations generally have a very short 'shelf-life' of only a few days, and furthermore, many thrombocytopenic patients who require chronic platelet transfusions become sensitized, necessitating HLA-matched platelet donors.

We have also successfully utilized these lentiviral vectors to achieve knockdown of HLA expression in hES cell-derived embryoid bodies (EB). The hES cell line H1 was mixed with concentrated lentiviral preparations of either the control vector or the HLA silencing shRNA vector, followed by washing and replating on DR4 mouse fibroblast feeders, and puromycin selection. 7 days later, individual colonies were isolated from the cultures and expanded into EBs. Immunostaining quantified by flow cytometry showed that control lentiviral vector-transduced hES cells could be successfully selected with puromycin and were almost completely GFP-positive, and after transduction were still able to undergo normal differentiation into EBs, which showed high expression of HLA. In contrast, after transduction with lentiviral vectors expressing HLA-targeted shRNA, embryoid bodies independently derived from individual hES cell colonies showed significantly decreased cell surface HLA levels ranging from approximately 30 to over 90 % reduction (Fig. 9.4b). The persistence of HLA knockdown effects in hES cell-derived cells further highlights the utility of lentiviral vectors in mediating long-term stable transduction.

9.9 Additional Strategies and Technologies for Genetic Modification of Stem Cells to Avoid Rejection

Other strategies to genetically engineer stem cells and stem cell-derived graft tissues can be envisaged and should be explored, both with regard to the targeted molecules, as well as the targeting methodology. A few such other possible strategies are suggested below.

9.9.1 Other Loss-of-Function Strategies

It may be useful to suppress other cellular proteins that elicit or potentiate host immune responses against mismatched donor antigens, e.g., suppression of HLA class II antigens or co-stimulatory molecules. This represents a loss-of-function strategy aimed at inhibition of immunoactivation, rather than evading immunorecognition.

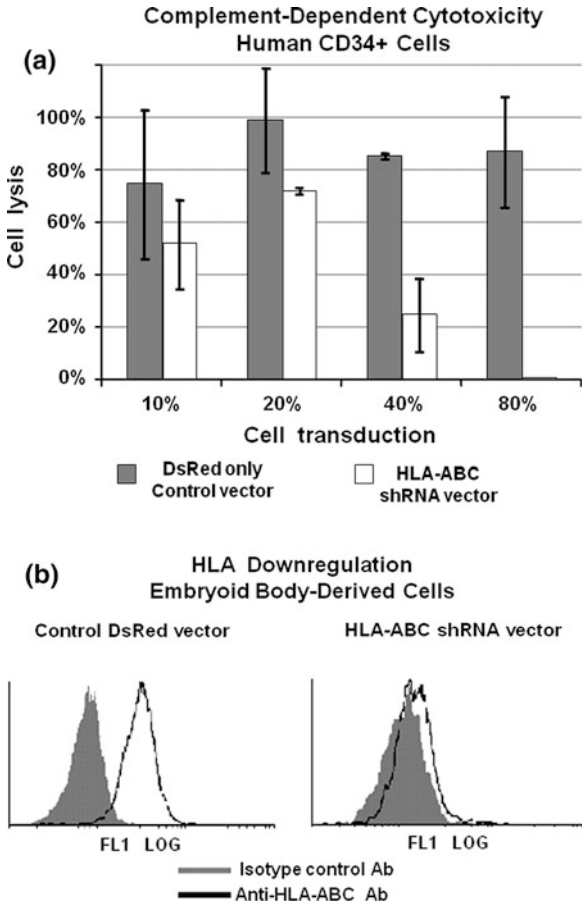

Fig. 9.4 Knockdown of HLA expression in primary human stem cell populations. Primary human $CD34^+$ hematopoietic stem cells or hES cells were transduced with either control lentiviral vector expressing the DsRed marker gene only, or lentiviral vector expressing the pan-class I HLA-ABC-specific shRNA in addition to DsRed. **a** Primary human $CD34^+$ hematopoietic stem cells were transduced with lentiviral vectors at a range of MOIs, and the transduction level at each MOI as determined by flow cytometry was grouped according to overall transduction level categories, as shown. Resistance to anti-HLA antibody-mediated lysis was measured by complement-dependent cytotoxicity (CDC) assay. Cell lysis values reported are the average results of reactions with at 5–12 different antibodies with specificities against the HLA type of the transduced $CD34^+$ cells. **b** Human ES cells were transduced with lentiviral vectors, and differentiated into EBs. Disaggregated EB cells were stained with a FITC-labeled antibody against HLA-ABC, and analyzed by flow cytometry. X-axis: FL1 (FITC) channel fluorescence intensity (log scale); Y-axis, number of events. The histograms show representative flow cytometric results for human ES cell-derived target cells transduced with DsRed only control vector (left panel) or HLA-ABC shRNA vector (right panel), stained with isotype control antibody (gray shaded histogram) or with anti-HLA class I antibody (open histogram)

HLA class II antigens on endothelial and epithelial cells of allograft tissues are up-regulated by inflammatory cytokines, particularly IFN-γ, produced during rejection responses. It has recently been recognized that the development of donor-specific antibodies directed against mismatched HLA class II antigens is correlated with chronic rejection and is a negative prognostic indicator for graft survival [56, 57]. Hence, down-regulating the ability of allograft tissues to induce HLA class II may be useful for preventing or ameliorating chronic rejection. Jaimes et al. [58] recently employed lentiviral shRNA vectors to knock down class II HLA-DR or HLA-DM mRNA levels in a monocytic leukemia cell line by up to 85 and 75 %, respectively, and reported that HLA-DM knockdown had only moderate functional effects on allogeneic T cell reactivity, but HLA-DR knockdown resulted in almost complete suppression of granzyme B up-regulation and significant reduction of IFN-γ secretion by $CD4^+$ T cells. However, HLA class II knockdown had no effect on cytotoxic responses of allogeneic $CD8^+$ T cells when co-cultured independently from the helper T cells [58], suggesting that knockdown of class II alone may not be sufficient, and that combination strategies may be required to fully protect allograft cells.

Co-stimulatory molecules such as B7.1 (CD80) and B7.2 (CD86) on APC are also up-regulated on endothelial and certain epithelial cells during inflammation, and this is thought to play an important role in autoimmunity as well as rejection of allografted organs such as lung and kidney [59, 60]. Therefore, engineering stem cells or stem cell-derived tissues to express siRNA against B7 family co-stimulators prior to transplantation, may also be worth pursuing as a strategy to evade immune rejection. One advantage of targeting co-stimulatory molecules is that these sequences are likely to be invariant in most individuals, and so individualized siRNA sequences do not need to be designed. Furthermore, apart from donor-derived APCs in allograft tissues, B7 co-stimulators are not normally expressed unless induced by inflammatory cytokines, so constitutive expression of siRNA specific for their (non-expressed) mRNAs in donor-derived non-APC cells should not have any functional effect until rejection responses occur. However, a potential disadvantage of this strategy is that generalized knockdown of co-stimulatory signaling in the allografted organ or tissues may later compromise normal immune function against pathogens. Furthermore, CD28 activation by B7 may also be essential for maintaining homeostasis of $CD4^+CD25^+$ Treg cells [61], so B7 knockdown might also have unintended negative effects on induction of tolerance to donor cells.

9.9.2 Gain-of-Function Strategies

Gain-of-function strategies aimed at up-regulation or overexpression of dominant-negative inhibitory proteins (e.g., HLA-G), transmembrane co-inhibitory factors (PD-L1, PD-L2), or secreted immunosuppressive factors (e.g., interleukin-10), may also cause inactivation or elimination of responding immune effector cells and thereby prove to be an equally effective countermeasure against immune rejection.

HLA-G is a non-classical, dominant-negative form of HLA that is normally expressed by extravillous cytotrophoblasts in the placenta, and is thought to be involved in inhibiting maternal rejection of paternal HLA expressed by the semiallogeneic fetus [62]. Notably, many types of cancer have been reported to up-regulate HLA-G, indicating its role in enabling tumors to escape immunosurveillance [63]. HLA-G levels correlate with graft survival in heart, kidney, liver, and lung transplantation [64–67], and it has been reported that both membrane-bound and soluble isoforms of HLA-G can protect even xenogeneic porcine endothelial cells against attack by human NK cells [68, 69].

Programmed Death 1 (PD-1) ligands are B7 family members that act as negative regulators of co-stimulatory signaling. It has been proposed that co-stimulatory blockade and/or co-inhibitory signaling could provide an approach to ameliorate allograft rejection, and it has been reported that porcine cells overexpressing PD-ligands could suppress human T-cell activation and expand Treg cells in xenogeneic mixed leukocyte reactions [70, 71]. Again, however, a potentially significant disadvantage is that generalized inhibition of co-stimulatory signaling in the allograft tissue may compromise normal immune function against pathogens. A similar concept is the use of gene transfer to introduce a soluble form of CTLA4 (Cytotoxic T-Lymphocyte Antigen 4; CD152) for blockade of CD28/B7 signaling, such as CTLA4-Ig [72]; this approach was reported to prolong allograft survival in animal models, although without induction of long-term tolerance, and the best results were obtained in combination with gene transfer of other immunosuppressive proteins such as TGF-β or CD40-Ig [73, 74].

Interleukin-10 (IL-10) is an anti-inflammatory cytokine, and since it is a secreted protein, overexpression of IL-10 has the potential to achieve more potent down-regulation of allogeneic responses through a paracrine effect that can extend beyond the immediately transduced cells. This would be of considerable benefit if high levels of ex vivo gene transfer cannot be readily achieved in certain types of stem cells. Unlike TGF-β which has undesirable fibrogenic activity in addition to immunosuppressive activity, IL-10 can suppress Th1 cells and APCs, inhibit production of inflammatory mediators, and induce antigen-specific Treg cells [75]. It has recently been reported that gene delivery of human IL-10 could achieve short-term improvement in pulmonary function and attenuated inflammation after ex vivo adenoviral gene transfer during normothermic perfusion of discarded human lungs and isolated pig lungs [76], as well as short-term reduction in inflammation after transbronchial instillation of aerosolized plasmid liposomes in a rat model of lung transplantation [77]. Given the short-term duration of both plasmid- and adenovirus-based gene expression, lentiviral vectors may again prove more advantageous for more stable expression, but persistent expression of IL-10 at high levels may compromise normal immune function against infectious pathogens. A potential solution might be to explore the use of inducible promoters to allow transgene expression to be switched off when necessary, but on the other hand, achieving reliable and consistent inducibility in primary cells is often problematic.

Microbial immunomodulatory proteins expressed by a variety of different viruses and bacteria naturally function to forestall immune recognition or inhibit

innate and adaptive immune responses. For example, HIV nef protein, HTLV p12 protein, and adenovirus E3 region 19 K protein, can function to bind and sequester HLA in infected cells, preventing class I antigen presentation [78–80]. The latter example is especially interesting, as a comparison of E3-19 K proteins from several adenovirus serotypes revealed differential affinities for various HLA alleles [81, 82], raising the possibility of selectively utilizing these proteins for allele-specific HLA down-regulation. Notably, adenoviral vector-mediated gene transfer of E3-19 K has been reported to prolong human xenograft survival transiently [83], and more recently, pancreatic islet cells engineered to express E3-19 K via lentiviral vector-mediated gene transfer have been reported to achieve prolonged correction of hyperglycemia after transplantation in allogeneic mice [84]. Other examples include cytomegalovirus (CMV), herpes simplex virus (HSV), human herpesviruses (HHV-6, HHV-7, HHV-8), Epstein-Barr virus (EBV), and poxviruses, which all carry multiple genes encoding immunosuppressive functions, including immune evasions that down-regulate HLA, receptor analogs that block immune activation pathways, as well as cytokine and chemokine analogs that mimic inhibitory factors such as IL-10 [85–93]. Interestingly, neurotropic viruses such as HSV, CMV, and rabies virus have also been reported to up-regulate endogenous HLA-G, perhaps via expression of viral IL-10 analogs, thus utilizing the host's own dominant-negative machinery to subvert immunorecognition [93, 94]. While these also represent foreign proteins which are themselves potential targets for immune attack, and host cells have frequently evolved countermeasures to prevent viral infection, the immune evasive activity of such proteins might be capable of overcoming allograft rejection as long as their expression levels are maintained in transplanted cells.

9.9.3 Additional Technologies for Sequence-Specific Gene Targeting

9.9.3.1 MicroRNA-Type shRNA Vectors

The latest design strategies for RNAi mimic the natural configuration of miRNAs [95], which represent endogenous short-hairpin RNA (shRNA) precursors of siRNA that are naturally present in non-coding sequences such as introns, and which are now known to play a major role in regulating the expression of multiple genes simultaneously [96, 97]. In newer construct designs, multiple shRNA sequences can be embedded into a single synthetic intron, which is allowed to undergo normal splicing and processing of the hairpin sequences by the intranuclear Drosha complex for use as microRNAs, which are then exported to the cytoplasmic Dicer complex for use in the RNA interference pathway [98]. Thus, this configuration enables multiple shRNAs to be produced from the same construct under normal microRNA regulation, and hence achieving enhanced efficiency and reduced cytotoxicity.

In this context, it should be noted that viruses also encode their own microRNAs (miRNAs) which can modulate host cell functions, including immune responses [99–101]. Hence, mining the 'RNAome' of various viruses for miRNAs involved in immunomodulation may also prove rewarding, and any such miRNAs identified can be directly exploited via incorporation into vectors for allograft cell engineering. Furthermore, as virally encoded miRNAs have undergone natural selection to be retained in the viral genome throughout the course of virus evolution, these regulatory sequences will also provide clues toward advancing our understanding of their targets, which should represent key cellular signaling pathways that can be manipulated to induce immune tolerance.

It should also be possible to achieve sequence-specific reduction of targeted mRNAs encoding HLA or other critical immunoregulatory proteins through the use of "classic" anti-sense mRNA or ribozymes (which mediate RNA-catalyzed cleavage of targeted sequences). However, the success rate of these older technologies is frequently quite variable, and they have largely been superseded by the use of RNAi.

9.9.3.2 Genome Editing by Targeted Nucleases

Highly promising new technologies have recently been developed for engineering modified transcription factor proteins to recognize and bind specific sequences in chromosomal DNA, thereby making it possible to introduce precise changes at the genomic level. For example, engineered zinc-finger nucleases (ZFN), consisting of sequence-specific zinc finger transcription factor domains fused to a *Fok I* endonuclease domain, can be used to generate a double-stranded break (DSB) at a single site within the DNA sequence of a target cell's genome. Cellular DSB repair pathways frequently introduce mutations into the targeted gene sequence during the repair process, resulting in knockout of functional gene expression [102], or a template strand can be introduced along with the ZFN to initiate gene correction by homologous recombination [103]. More recently, transcription activator-like effector nucleases (TALENs), based on pathogenic bacteria from plants, also show considerable promise as a similar gene-targeting system exhibiting highly modular and readily manipulable DNA sequence-specific binding [104–106]. For both ZFN- and TALEN-based gene knockout approaches, once genome modification has been achieved, the ZFN or TALEN constructs are no longer needed, so they only need to be expressed transiently in the stem cell, a so-called "hit-and-run" strategy. Thus, unlike the case with RNAi strategies, long-term transgene expression from integrating vectors such as lentivirus is not needed, thus mitigating potential issues of vector-related genotoxicity. On the other hand, the genomic modifications introduced are permanent, and may include some level of "off-target" effects resulting in mutations to unintended genome sequences, a topic of ongoing scrutiny and efforts to improve target specificity [107–109].

9.10 Designing "Universal" Stem Cell Lines: Perspective

As demonstrated above, combining the state-of-the-art technologies of siRNA silencing and lentivirus vector-mediated genetic engineering now offers the potential to achieve immunological evasion by genetically reprograming allogeneic cells, and may be as effective as utilization of powerful non-specific immunosuppressive agents. General down-regulation of HLA class I expression may make it possible to create "universal" hES cell-derived donor cells; in this context, it may be advantageous that HLA knockdown with these particular pan-class I shRNA constructs did not result in complete loss of expression even at high MOI, as this may be why sensitivity to non-HLA-restricted killer cell activity was not observed. Alternatively, as noted, down-regulation of specific HLA alleles by precise siRNA targeting may expand the histocompatibility and utility of existing hES cell-derived cells and tissues by nullifying certain classes of HLA sequences, and thus making it easier to find matches with the remaining HLA sequences.

Ex vivo genetic engineering of stem cells and stem cell-derived tissues represents a fundamental shift in the approach for achieving graft survival, by modifying the properties of the transplanted cells rather than immune suppressing the recipient. While efficient gene transfer to entire solid organs remains a technical hurdle, application of this strategy can be readily envisaged for ex vivo transduction of cellular transplants in which HLA matching is a rate-limiting factor, such as hematopoietic stem cell transplants, skin grafts, and pancreatic islet cell transplants. Furthermore, immuno-evasive or immune-modulatory gene transfer technology may be applied to reduce the development of immunogenicity in pluripotent stem cells or lineage-specific adult stem cells, or alternatively, in more differentiated cells and tissues derived thereof, before or after transplantation in non-autologous settings. By the same token, these various strategies and technologies are available for further development of effective approaches to engineer "universal" stem cells, and show considerable promise as a generalized solution to the immunological barriers confronting regenerative medicine.

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Chapter 10
The Immunosuppressive Properties of Adult Stem Cells: Mesenchymal Stem Cells as a Case Study

Francesco Dazzi and Ilaria Marigo

Abstract The last few years have witnessed an enormous interest in mesenchymal stem cells (MSC). Their popularity is probably to be ascribed partly to the relative ease with which they can be generated but also to the many reports attributing them a variety of therapeutic activities ranging from pluripotency to immunosuppression. The large volume of functional data produced during the last decade, although sometimes contradictory, has opened up new avenues but has driven the efforts away from identifying solid criteria to define the identity of MSC and their progenitors: this task has been thwarted by the absence of good in vivo models to test their reconstituting and differentiation capacity similar to those available for the study of hematopoietic stem cells.

10.1 Mesenchymal Stem Cells: A Terminology in Need of Refinement

According to the standard definition of a stem cell, mesenchymal stem cells (MSC) are a population of multipotent cells capable of self-renewal and differentiation into cells of the mesenchymal lineage including osteocytes, chondrocytes and adipocytes. They also give rise to a stromal population which supports hematopoiesis, also named as hematopoietic niche cells. However, in real life, most aspects of MSC biology, including their ontogeny, anatomical location in the bone marrow and in vivo functions have not been fully elucidated. Attempts to clarify these issues have produced confounding results, principally because many

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researchers have employed different methods to isolate and expand MSC, assess their differentiation potential and capacity for self-renewal.

Currently, the most common basic approach to isolate MSC relies on the selection of adherent mononuclear cells from adult bone marrow or adipose tissue. Our knowledge of MSC is primarily confined to the characterisation of cultured cells with in vitro self-renewal capacity. The population thus isolated expresses a number of markers (CD73, CD90, CD105 and the absence of hematopoietic markers) shared by other mesenchymal stromal cells, differing in progenitor content. Therefore, the acronym MSC is currently used to refer to a heterogeneous population of stromal cells, a large proportion of which are mesenchymal progenitors [1]. Although such a definition has its relevance to facilitate a consensus as to the cell type employed for therapeutic applications, it is of little use to understand their properties and hierarchy.

Depending on the study, cultured MSC express different lineage-specific antigens, adhesion molecules and growth factor receptors [2], thus outlining the intrinsic heterogeneity of MSC preparations. The long-term cultures, usually employed before their characterisation, may confer the cells different characteristics from their in vivo state. Despite remaining multipotent, cultured MSC show poor tissue tropism when transplanted [3, 4]. Not all results are in agreement with this because others have found that primary MSC show efficient seeding of the bone marrow [5].

A first attempt to identify MSC-specific markers recognised stage-specific embryonic antigen-1 (SSEA-1) expressing bone marrow stromal cells in adult mice as precursor cells with a high capacity to differentiate not only to several mesenchymal cell types but also to cell types of different embryonic origins, thus suggesting that this marker may identify a pre-MSC stage with characteristics similar to the previously described multipotent adult progenitor cells (MAPC) [6].

More recently, a study used phenotypic, morphological and functional criteria to identify and prospectively isolate a subset of MSC ($PDGFR\alpha^{+}Sca\text{-}1^{+}CD45^{-}TER119^{-}$) [7] from adult mouse bone marrow. The cells thus isolated exhibited extensive proliferative activity without signs of senescence, and could differentiate into hematopoietic niche cells, osteoblasts and adipocytes after in vivo transplantation [8]. Although it seems that MSC generally reside in the perivascular region [9], their differentiation options seem to be selected by the local tissue. MSC in the bone marrow mainly differentiate into niche cells, while the cells localised to adipose tissue tend to follow adipocyte differentiation. Further markers, like CD146, appear to specifically define a progenitor, lower in the hierarchical structure, committed to niche-type cells [10].

10.2 Origin and Sourcing of MSC

The notion of tissue-specific MSC function [10] has been recently supported by an elegant study investigating MSC differentiation from ES cell cultures. The work showed that $Sox1^{+}$ neuroepithelial cells supply the earliest wave of MSC

differentiation, which occurs during embryogenesis but is later replaced by MSC from other origins in postnatal development [11]. Therefore, the data currently available suggest that 'mesenchymopoiesis' is characterised by the presence of primitive stem cells confined in a tissue—possibly the bone marrow—and a series of progenitors with more specialised functions appropriate to the organ/tissue of primary homing.

Pre-MSC type cells with characteristics of pluripotency have been isolated in the bone marrow or in fetal/perinatal tissues. Good examples are MAPC which differentiate into various lineages in vitro using defined cytokine combinations, and when transplanted they directly contribute to hematopoiesis in vivo and generate long-term repopulating hematopoietic stem cells and the full repertoire of hematopoietic progenitors [6]. Cells with similar properties have been described in human bone marrow [12] and in human cord blood [13]. Unrestricted somatic stem cells of cord blood origin share several markers with MSC and can be differentiated in vitro and in vivo into osteoblasts, chondroblasts, adipocytes as well as hematopoietic and neural cells. A number of cells with mesenchymal progenitor activity have been detected in various tissues other than bone marrow. MSC-like cells can be isolated and expanded from the adipose tissue [14], synovial membrane [15, 16] and even in the peripheral blood. Fetal tissues are particularly enriched for MSC, including liver, bone marrow and the amniotic fluid.

Within these considerations it should not be forgotten that fibroblasts are often indistinguishable from what is currently defined as MSC, especially those derived from the bone marrow [17]. Fibroblasts exist in virtually every organ in the body. They are defined as adherent cells, which are not endothelial, epithelial or hematopoietic in origin, and which have the capacity to synthesise and remodel the extracellular matrix. Similar to MSC, fibroblasts have been directly shown to play roles in regulating immunological tolerance, organ development, wound healing, inflammation and fibrosis. Their functions differ according to the tissue from which they are derived. There is also evidence that dermal fibroblasts can differentiate in vitro into cells of the mesenchymal lineage, thus further supporting the overlap with MSC.

Lastly, this brief review of the various entities that can be defined under the general terminology of MSC should mention fibrocytes, a circulating, bone marrow-derived cell which, despite being of hematopoietic origin, has the ability to adopt a mesenchymal phenotype [18]. This cell type exhibits features of both fibroblasts and monocytes, and this unique combination allows them to be identified by a specific combination of markers. They express CD34, the hematopoietic marker CD45, and produce components of the connective tissue matrix including collagen type-1. Similar to MSC, fibrocytes are capable of differentiating into fibroblasts and myofibroblasts, as well as adipocytes.

The significant overlap between these populations highlights our poor understanding of the hierarchy, but also suggests a common functional activity within the lineage.

10.3 MSC Exert a Potent Immunosuppressive Activity

Despite their competence in acting as antigen presenting cells, MSC have been observed to exhibit a unique property, namely to produce potent immunosuppressive effects. Such an ability has been reported in MSC derived from humans, rodents and primates, and its magnitude is superior to any of the immunoregulatory networks known thus far, including regulatory T cells (Treg) [19] and immunosuppressive monocytes/macrophages [20]. When MSC are added to cultures of T cells stimulated with any sort of agent—not only their cognate peptide but also to mitogenic and polyclonal stimuli—they inhibit their proliferative responses. The immunosuppressive effect is very potent because it is still prominent when MSC and responding lymphocytes are at a 1:50 ratio [21].

Tolerance induction has been well documented to be accomplished by professional antigen presenting cells, whereby immature dendritic cells (DC) specifically present the antigen to T cells in an inefficient, tolerogenic fashion [22]. However, in the case of MSC, the 'tolerogenic' activity—or more precisely the induction of unresponsiveness—is not antigen-dependent, because it does not require the expression of major histocompatibility complex (MHC) molecules on the surface. In fact, the immunosuppressive activity on MHC class I-restricted $CD8^+$ T cells can still be observed using MSC deficient in the genes encoding MHC class I molecules [21]. Furthermore, the use of MSC from third-party donors fully mismatched for the MHC haplotype of the responder T cells are similarly effective [23]. Therefore, MSC-induced unresponsiveness is not antigen-specific and it also lacks any selectivity. In fact, MSC are equally effective at inhibiting the proliferation of memory and naïve T cells [21], do not preferentially affect $CD4^+$ or $CD8^+$ subsets [24] and have similar effects on B cell proliferation.

10.4 How MSC Affect T Cells

The characterisation of MSC-induced anergic T cells has provided interesting information to help to elucidate the mechanisms underlying MSC-mediated immunosuppression and to suggest a possible physiological significance of this phenomenon. Whilst MSC do not affect T cell activation, they potently interfere with cell proliferation. T cells, stimulated in the presence of MSC, are arrested at the G_0/G_1 phase of cell cycle as a result of inhibition of cyclin D2—the master regulator of cell cycle entry—as well as the upregulation of $p27^{kip}$ [24]. Since the effector functions are only partially impaired, MSC induce an unresponsive T cell profile that is fully consistent with 'division arrest anergy' [25], a phenotype frequently identified in tumour-reactive T cells in patients with malignancies whereby cell proliferation is profoundly inhibited despite partial conservation of effector functions [26].

The capacity of T cells to proliferate appears to be rescued by the addition of exogenous interleukin-2 (IL-2) [27], thus indicating that the inhibition is reversible. In vivo studies conducted in experimental autoimmune encephalomyelitis (EAE) indicate that T cells, obtained from MSC treated mice, exhibit an impaired proliferative response to myelin oligodendrocyte glycoprotein (MOG) peptides as well as to mitogenic stimulation [28]. Although the suppression of this response was abrogated in vitro in the presence of IL-2, the adoptive transfer of encephalitogenic T cells activated by the cognate antigen in the presence of MSC induced only a milder form of the disease as compared to that induced by untreated encephalitogenic T cells [29].

The evidence that T cells are only temporarily inhibited in their proliferative/functional capacity suggests that MSC do not cause cell death. However, there is no conclusive evidence to support this contention. It appears that MSCs prevent activation induced cell death in murine T cells [16, 30] even if one of the mechanisms involved in arresting cell proliferation is the engagement of the inhibitory molecule programmed death 1 (PD-1) [31]. In humans, the prominent role of indoleamine 2,3-dioxygenase (IDO) in the T cell-MSC interaction (described below) might justify the opposing observation that MSC inhibit T cell proliferation by inducing the apoptosis of activated T cells, but have no effect on resting T cells [32].

10.5 MSC Activity Impacts on Other Immune Effectors

The immunosuppressive effects of MSC is not confined to T cells. MSC inhibit IL-2-induced proliferation of resting natural killer (NK) cells, prevent the induction of their effector functions, but interfere only partially with the proliferation of activated NK cells [33, 34]. However, MSC are not resistant to NK cell-mediated killing provided the NK cells have been pre-activated with IL-2. Accordingly, MSC express the natural ligands for the activating NK receptors NKp30, NKG2D and DNAM-1. If MSC are pre-treated with IFN-γ they upregulate HLA class I molecules at their surface and as a consequence become resistant to NK-mediated lysis.

Human MSC have also been shown to inhibit B cell function because immunoglobulin production can be impaired and the expression of certain chemokine receptors and hence the response to specific chemokines is negatively affected by MSC [35]. However, further studies have challenged this view. When the influence of bone marrow MSC was evaluated on highly purified B cell subsets, they were observed to promote proliferation and differentiation into immunoglobulin-secreting cells of transitional and naive B cells stimulated with an agonist of Toll-like receptor (TLR) 9, in the absence of B cell receptor triggering. Under these conditions, MSC enhanced proliferation and differentiation into the plasma cells of the memory B cell populations [36]. Although the stimuli utilised in this study are different from those in previous reports, the results suggest that the MSC immunosuppressive activity is

regulated and not necessarily indiscriminate. MSC contribute to the bone marrow reticular niche, where mature B cells and long-lived plasma cells are maintained [37] and in the thymus they effect a similar function [38].

10.6 MSC and Antigen Presenting Cells

The immunosuppressive properties of MSC can also target antigen-presenting cells (APC). The same effects exerted on cell cycle progression in T cells have been documented to affect monocytes. MSC inhibit the differentiation of DC, induced by interleukin-4 and granulocyte macrophage-colony stimulating factor, from monocytes or $CD34^+$ cells [39–41]. When exposed to MSC, DC precursors do not acquire a mature phenotype and are impaired in their APC function because their ability to stimulate alloresponses is much reduced. Furthermore, several pieces of evidence show that these 'aborted' DC acquire regulatory features. Human MSC induce the generation of DC producing large amounts of IL-10 and impaired in IL-12 production. These findings highlight an important concept which is that not only do MSC act directly on immune effectors but also generate regulatory APC with T cell suppressive properties [42, 43]. Similar data have been confirmed in mouse whereby MSC, whilst promoting the proliferation of mature DC, induce a decrease in the expression of co-stimulatory molecules. Under the influence of MSC, mature DC differentiate into a novel Jagged-2-dependent regulatory DC [44].

10.7 Molecular Mechanisms of MSC Mediated Immune Suppression

A variety of molecular mechanisms has been described that could account for the immunoregulatory activities of MSC. Initially, there were contrasting results as to whether such activities were cell contact dependent or mediated by soluble factors. The conundrum was then resolved when the candidate molecules became clearer because the majority of them, despite being soluble factors, require MSC and the target cells to be in close proximity because of the short-term activity and the short distance availability of these molecules. However, there is also evidence that a cell contact-mediated interaction may be required as a first step, as suggested by studies showing that the inhibition of T cell proliferation by MSC requires the engagement of the inhibitory molecule PD-1 [31].

The molecules identified thus far are not necessarily produced by MSC under the same conditions, because different microenvironments preferentially drive the production of different molecules. By using neutralising monoclonal antibodies, early observations identified transforming growth factor-β (TGF-β) and hepatocyte growth factor as the mediators of the human MSC effects [27]. TGF-β has very

recently been implicated in the beneficial effects mediated by MSC in suppressing allergic responses in a mouse model of ragweed-induced asthma [45].

Further studies in the human have demonstrated that MSC utilise indoleamine 2,3-dioxygenase (IDO). IDO is an intracellular enzyme that initiates the first and rate-limiting step of tryptophan breakdown along the kynurenine pathway. Whilst its role was thought to be mainly confined to antimicrobial activity by limiting availability of essential nutrients in the environment, it was later discovered that IDO has a central role in mediating immune privilege and preventing rejection of the allogeneic fetus during pregnancy. Tryptophan depletion causes a rise in the level of uncharged tRNA in T cells, resulting in activation of the amino acid-sensitive general control non-depressible 2 (GCN2) stress kinase pathway with consequent cell cycle arrest and anergy [46, 47]. Certain downstream tryptophan-derived metabolites exhibit similar effects on T cells [48]. The functional phenotype of the T cells exposed to IDO is in fact very close to what has been described in T cells that have been in contact with MSC [24]. Moreover, IDO production is highly sensitive to the presence of interferon-γ (IFN-γ) which, as we will discuss later, plays a prominent role in driving the immunosuppressive activity of MSC. Despite several pieces of evidence supporting a fundamental role for IDO in human MSC, confirmation that it has an equal function to that reported for murine MSC has yet to be obtained [49].

In contrast to human MSC, mouse MSC utilise nitric oxide (NO), at least under the culture conditions that promote IDO in human MSC [50]. Studies have reported that MSC suppress Stat5 phosphorylation and proliferation in T cells via a pathway involving NO. The induction of inducible NO synthase (iNOS) was readily detected in MSC and specific inhibitor of iNOS reversed the suppression of T cell proliferation [49]. Nitric oxide at high concentrations is known to inhibit T cell responses [51], but since it diffuses rapidly from its source, the active form must be available in close proximity to the target cell, thus justifying the ambiguity of the conclusions regarding the need for cell contact in MSC-mediated immunosuppression. The role of NO was subsequently confirmed by other studies which correlated the production of iNOS to the presence in culture of at least three inflammatory molecules, namely IFN-γ, tumour necrosis factor-α (TNF-α) and IL-1α or IL-1β.

Similar to the involvement of NO, other studies have proposed heme oxygenase-1 (HO-1) as a further mechanism of MSC-mediated immunosuppression. HO-1 has anti-inflammatory and immunosuppressive activities and can mediate the effect of NO [52]. HO-1 has been shown to be crucial in human MSC, whilst co-operating with NO in rat MSC. The important role of these molecules was also demonstrated in vivo because the administration of HO-1 and iNOS inhibitors reversed the ability of MSC to prolong cardiac allograft survival in a rat model [53].

An important molecule, reported in several studies, is prostaglandin E_2 (PGE_2). It was initially observed that human MSC produced elevated levels of PGE_2 when in culture with immune cells, and that PGE_2 inhibitors partially restored MSC immune modulation on T cells [42] and NK cells [34]. It has subsequently been

described that PGE_2 is one of the major effectors of MSC therapeutic activity, whereby MSC have been shown to reprogram macrophages by releasing PGE_2 that impacts on macrophages via their prostaglandin EP2 and EP4 receptors [54]. In this model, in which MSC were administered to a mouse model of acute sepsis, MSC-recruited macrophages were fundamental for the beneficial effect that was ultimately mediated by IL-10 [54].

HLA-G protein expression has been found to be constitutively expressed in MSC and anti-HLA-G blocking antibody partly restores lymphocyte proliferation in the presence of MSC [55]. It was then noted that surface HLA-G was not involved in their protection from cytotoxic T cell-mediated lysis and that the soluble form, contained in the supernatant of MSC cultures was responsible for the inhibitory effect [56]. HLA-G is a non-classic MHC class I molecule that functions as an immune-tolerogenic molecule with restricted tissue expression. HLA-G has a direct inhibitory effect on immune responses but there is also increasing evidence that it can induce the generation of suppressor/regulatory cells [57]. Accordingly, it has been shown that MSC secrete, in an IL-10 independent fashion, the soluble isoform HLA-G5 that not only suppresses allogeneic T cell proliferation but also determines the expansion of $CD4^+CD25^{hi}FoxP3^+$ Treg cells [58]. Like IDO, HLA-G has an important role in immunological tolerance to the fetus, thus suggesting that MSC-mediated immunosuppressive activity should be considered a modality of 'innate tolerance', a function shared by other cells of different ontogenetic origin rather than a function of a specific cell type. In fact, monocytes/macrophages and other cells of myeloid origin can exhibit, under particular circumstances, almost identical functions and this important concept will be described in more detail later in this chapter.

Finally, other mechanisms have been reported that involve IL-6 [59] or the production of insulin growth factor binding protein independently of IDO [60].

10.8 Are MSC Immunoprivileged?

The prominent immunosuppressive activities would suggest that MSC represent an immunoprivileged cell type because the environment surrounding MSC would impair histoincompatible MSC to be rejected. This property would have a huge impact on MSC transplantation and tissue repair strategies, because MSC could be expanded from a third-party donor, irrespective of the histocompatibility of the recipient, and made available to any patients who could benefit from them. Unfortunately, this does not seem to be the case. It was found that MSC exhibit phagocytic properties and, in the presence of low levels of interferon-γ (IFN-γ), upregulate MHC class II molecules. Under these circumstances, MSC can function as APC and stimulate $CD4^+$ T cells to recall antigens [61]. Human MSC can process and present HLA class I-restricted virus- or tumour-encoded antigens to $CD8^+$ cytotoxic T lymphocytes (CTL) although with a limited efficiency, probably related to an incomplete antigen processing machinery [56]. Although capable of

generating CTL, MSC are not susceptible to their lysis and this resistance has been correlated to the release of soluble HLA-G molecules [56].

Their immunogenicity has also been demonstrated in vivo using an elegant model. Murine MSC, engineered to release erythropoietin were implanted subcutaneously in either syngeneic or MHC-mismatched mice and hematocrit was monitored as a reporter of MSC graft survival. Whilst in syngeneic recipients, the hematocrit increased for more than 200 days, in allogeneic mice, it rose only transiently with complete refractoriness after repeat implantations [62]. Similar findings were reported in a bone marrow transplantation model. Sublethally irradiated recipients received allogeneic bone marrow with or without host (syngeneic) or donor (allogeneic) MSC. Whilst host MSC enhanced long-term donor hematopoietic engraftment, the infusion of donor MSC was associated with the rejection of allogeneic donor bone marrow cells [40]. More recently, the effect of MSC infusion to induce tolerance to allogeneic skin grafts was examined in rats. The administration of donor MSC in cyclosporin-treated rats increased skin allograft survival but not if rats did not also receive cyclosporin, thus indicating that allogeneic MSC could accelerate graft rejection and suggesting the need for MHC-identical MSC [63].

These data have to be reconciled with the potent immunosuppression displayed by MSC: how can MSC be immunogenic if they suppress immune responses? There may also be a discrepancy between the murine and human MSC characteristics. The results from the current clinical studies suggest that the therapeutic immunosuppressive activities do not vary with the type of donor used to generate MSC; the clinical efficacy being similar if a fully compatible donor or a third-party donor is used to produce MSC [64]. One hypothesis that could resolve the dilemma is that the immunosuppressive activity in vivo relies on two separate components: one is the direct effect mediated by the MSC, the second is the recruitment of the recipient's immunoregulatory networks, which continue the therapeutic effect without the need for the persistence of MSC in the local microenvironment. However, it remains to be clarified why MSC have been proposed to control graft rejection when they are rejected themselves.

10.9 MSC Require 'Licensing' to Acquire Immunosuppressive Properties

The immunosuppressive properties of MSC are not constitutive, but require a 'licensing' step capable of promoting the immunosuppressive properties. We described before that the majority of the factors involved in such an activity are soluble factors. However, if the supernatant of MSC cultures is transferred to a culture in which T cells are being stimulated, no inhibition of immune responses can be detected. However, if the T cell cultures and MSC are co-cultivated but separated by a membrane which allows the diffusion of soluble factors (transwell)

the inhibitory activity is almost as effective as when MSC and T cells are in direct contact. This suggests that in the T cell cultures there are molecules that are necessary for the MSC functions to be enacted. In fact, if the supernatants come from cultures in which MSC are in the presence of activated T cells, then these supernatants contain immunosuppressive activity [16]. The concept that MSC require a 'licensing' step to become activated has important implications in the understanding of their normal functions and for therapeutic exploitation [65] that we will discuss later in the chapter.

The nature of the 'licensing' signals are strictly related to inflammation. The first report showing this link was based on the in vitro observation that IFNγ is necessary to promote MSC mediated immunosuppression. In this study the suppressive activity of MSC on $CD4^+$ and $CD8^+$ T cells was reversed following the addition of anti-IFN-γ receptor neutralizing antibodies to the cultures [66]. These data are consistent with the fact that IFN-γ is a potent inducer of IDO, which is one of the main mediators of MSC immunosuppression. If these findings were reported in humans, similar findings were also observed in mice whereby the 'licensing' signal was identified as a combination of IFN-γ, TNF-α and IL-1β [67].

The concept of 'licensing' should be considered more widely, because it does not necessarily imply the conferment of inhibitory properties. Early studies into the immunological attributes of MSC observed that whilst high numbers of MSC suppress alloreactive T cells, very low numbers stimulate lymphocyte proliferation [23]. The ability to inhibit or stimulate T cell alloresponses seems to be independent of the MHC, since the use of third-party MSC or MSC autologous to the responder T cells did not modify the outcome. Although incomplete, this observation identified a novel concept in the immunology of MSC, the possibility that their immunological properties can be 'polarised' either towards an immunosuppressive/anti-inflammatory or an immunostimulating/pro-inflammatory phenotype. One of the key molecular mechanisms involved in the immune polarization is the stimulation of TLRs. MSC express almost all TLRs and they are functional, as confirmed by nuclear factor kappa B (NF-κB) translocation in response to the corresponding ligand. Pam3Cys, a prototypic TLR-2 ligand has been shown to increase MSC proliferation, and inhibit their differentiation into osteogenic, adipogenic, and chondrogenic cells whilst not affecting their immunosuppressive activity [68]. On the contrary, ligation of TLR3 and TLR4 inhibits the ability of MSC to suppress T cell responses, without influencing their differentiation potential [69]. Accordingly, more recent studies have observed that TLR activation of MSC results in the chemoattraction of innate immune cells both in vitro and in vivo. In addition, TLR activation combined with IFN-γ priming increases the efficiency of MSC to function as APC [70].

Therefore, MSC are constitutively immunologically neutral but can acquire pro- or anti-inflammatory properties depending on the surrounding environment. This concept also explains the apparently contrasting findings reported about the effect of MSC on B lymphocytes. The stimuli used to activate and/or differentiate B cell functions differ from those utilized for T cells with consequences on the cytokine pattern produced as a consequence of B cell activation. In the presence of

some B cell activators (CpG) MSC stimulate B cell proliferation and immunoglobulin production [36], whilst the presence of IFN-γ enables MSC to inhibit B cell proliferation [69]. The in vivo implications are crucial and will be discussed in the section on the therapeutic use of MSC.

10.10 MSC Recruit Further Immunoregulatory Networks

MSC-mediated immunosuppression is not confined to a direct action on the effector cells on the immune response, but also results from the recruitment of other immunoregulatory networks. MSCs communicate under a variety of circumstances with cells of the monocyte-macrophage lineage. As reviewed by Gordon and Taylor [71], these cells display remarkable plasticity and, in response to environmental signals, undergo different forms of polarised activation and can play a crucial role in immune defense, modulation and resolution of inflammation and tissue remodelling. They do so by phagocytosis, antigen processing and presentation, and by cytokine production. Monocytes are known to originate in the bone marrow from a common myeloid progenitor that is shared with neutrophils, and they are then released into the peripheral blood, where they circulate for several days before entering tissues. Inflammatory monocytes defined as $CCR2^{+}Ly6C^{+}$ monocytes are recruited and differentiate into macrophages at the site of the inflammatory lesion which showed the ability of these macrophages to acquire distinct phenotypes and physiological activities when elicited with different cytokines. In fact, IFN-γ and LPS induce a classical activation, with macrophages exhibiting high microbicidal activity, production of reactive oxygen species (ROS), as well as secretion of pro-inflammatory cytokines and activation of cellular immunity. By contrast, IL-4, IL-10, IL-13 or TGF-β induce an alternative activation that promotes tissue repair, humoral immunity and suppresses inflammation. It remains to be demonstrated whether these distinct activation states also exist in vivo or if there are a continuum of responses elicited by different inflammatory environments [71].

There is evidence that MSC polarise monocytes/macrophages to acquire an immunosuppressive profile. Murine MSC co-cultured with peritoneal macrophages stimulated with LPS induce a switch in macrophages function towards an inhibitory profile characterised by the production of anti-inflammatory cytokines, the ability to phagocytose apoptotic cells and an increase in their susceptibility to infection by the intracellular pathogen *T. cruzi*. This regulatory profile is induced by the release of PGE_2 from MSC [72]. Human bone marrow-derived MSC induce steady-state peripheral blood monocyte-derived-macrophage to acquire a peculiar phenotype characterised by an IL-10^{high}, IL-12^{low}, IL-6^{high}, and TNF-α^{low} cytokine profile with high phagocytic activity. This novel type of alternatively activated macrophages designated M2 macrophages (or MSC-educated macrophages, MEM) could exert an anti-inflammatory role and promote tissue repair [73]. Moreover, Nemeth and colleagues showed that the injection of bone marrow MSC

in a murine model of sepsis induced a higher secretion of IL-10 anti-inflammatory cytokines from macrophages due to an increased release of PGE_2 from these cells [54].

Other cells of myeloid/monocyte origin may play a role in the immunoregulatory network activated from MSC. Accumulating evidence suggests that a heterogeneous population of immature myeloid cells with immunoregulatory activity called myeloid-derived suppressor cells (MDSC) is expanded in various pathological conditions. These cells, comprise polymorphonuclear and mononuclear cells at different stages of maturation, defined in mice by surface expression of CD11b and Gr-1 antigens but weak expression of other markers of mature myeloid cells, with a high potential to suppress immune responses in vitro and in vivo [74]. Although most of the current information on MDSCs in immune responses have been derived from studies in the cancer field, MDSCs also regulate immune responses in bacterial and parasitic infections, acute and chronic inflammation, traumatic stress, surgical sepsis and transplantation [75–77]. On the basis of the differing expression of Gr-1, Ly6C and Ly6G molecules, a few groups have recently identified and separated MDSC subsets from the spleen of tumour bearing mice: a polymorphonuclear MDSC fraction ($CD11b^+Gr\text{-}1^{high}Ly6G^+Ly6C^{low/int}$) and a mononuclear MDSC fraction ($CD11b^+Gr\text{-}1^{int}Ly6G^-Ly6C^{high}$). In different organs and under diverse inflammatory and neoplastic conditions, the proportional composition of these subpopulations changes, although the role of the monocytic fraction appears prominent in restraining immune responses in mice [77, 78].

Furthermore, there are several lines of evidence supporting the notion that MSC activate and expand regulatory T cells (Treg), although Treg themselves are not required as a unique component to effect MSC immunosuppressive activity [24, 42]. $CD4^+$ Tregs are the main effectors of immunological tolerance in adult life. Tregs can be divided into 'naturally occurring' thymus-derived Tregs and those generated in the periphery known as 'adaptive or induced' Tregs. In general, Tregs can be reasonably identified as expressing the IL-2 receptor α-chain (CD25) and the forkhead box P3 (FoxP3) transcription factor [79, 80]. The analysis of T cells cultivated in the presence of MSC showed a heterogeneous population of $CD4^+$ and $CD8^+$ cells expressing variable levels of CTLA4, glucocorticoid-induced tumour necrosis factor receptor (GITR) and FoxP3 [81]. MSC-mediated Treg expansion has been widely documented in several in vivo systems, including experimental arthritis [82], breast cancer [83], asthma [45], diabetes [84] and EAE [28]. Noteworthy, in a heart transplant model, both the induction and maintenance phase of donor-derived MSC-induced tolerance were associated with donor-specific $CD4^+CD25^+Foxp3^+$ Treg expansion that impaired anti-donor Th1 activity. In fact, the adoptive transfer of splenocytes from tolerant mice prevented the rejection of fully MHC-mismatched donor-specific secondary allografts but not of third-party grafts [85]. A variety of mechanisms can account for Treg expansion. Not only do MSC secrete or express molecules like TGF-β, HLA-G or IDO that directly activate and expand Tregs, they also recruit immunoregulatory networks that actively engage Tregs by producing tolerogenic cytokines. All these pathways have been described previously in this chapter.

Whether the amplification of these networks by MSC is essential for immunosuppression in vivo remains to be clarified. However, MSC, MDSC, tumour-infiltrating macrophages and Tregs constitute an immunoregulatory network which is detectable in various pathological and cancerous conditions [86–89], thus supporting the notion of a functional compartment effecting tissue-restricted immune tolerance.

10.11 MSC Mediated Immunomodulation for Therapeutics: Lessons from Animal Models

There are probably very few areas in pathology in which MSC have not been tested with a view of treating a variety of diseases of completely different origin, from those caused by excessive immune responses to those resulting from a toxic or ischaemic insult to the tissue. We will review some of the experimental evidence obtained in pre-clinical models concerning the use of MSC to control immune-mediated diseases and then summarise the experience in the clinical setting.

The first evidence that MSC could exert an immunosuppressive activity was derived from an in vivo model of skin grafting in non-human primates. It was shown that in vivo administration of MSC prolonged allogeneic skin graft survival when compared to control animals receiving no MSC [90]. The results were confirmed more recently in rats whereby MSC infusions increased skin allograft survival, although only if persisting for some time [63]. A number of studies using MSC for transplantation have often found that if MSC are derived from a third-party donor, the beneficial effects can only be obtained in the concomitant presence of drug-induced immunosuppression (like cyclosporine or mycophenolate) [91, 92]. Consistent with these results are the findings obtained from islet transplantation. The use of MSC has been tested in a rat model of streptozotocin-induced diabetes with a view of producing hematopoietic chimerism concomitant with allogeneic islet transplantation. Following a conditioning regimen with anti-lymphocyte serum and 5 Gy total body irradiation, diabetic rats received an intraportal infusion of allogeneic MSC, bone marrow cells and islets. About-two thirds of recipients infused with MSC developed stable mixed chimerism and persistent islet engraftment [93]. Utilising MSC from an autologous source appears to circumvent the problem and deliver sufficient clinical activity [94]. Finally, the experience in experimental renal and liver transplantation is limited but appears to reiterate the partial benefit of MSC in facilitating engraftment [95, 96].

Sepsis is a severe disease, the pathogenesis of which is related to a bi-phasic immune response. Patients can die either early because of the initial overwhelming inflammatory response to the microorganism, or subsequently because of the profoundly delayed immune reconstitution. Bone marrow MSC, given around the initiation of sepsis, were found to increase the survival of recipient mice and

the effect was associated with reprogramming of monocytes to induce the production of large amount of IL-10 and decrease the concentrations of TNFα and IL-6 [54]. The results were contemporarily demonstrated with using human adipose-derived MSC in the same mouse model [97].

Very encouraging results have been obtained in the field of autoimmune diseases. MSC have been shown to delay the onset of diabetes in non-obese diabetic mice (NOD) if the MSC were derived from diabetic resistant strains (i.e., non-obese resistant mice or BALB/c), but not from NOD mice [98]. The systemic infusion of adipose tissue derived MSC was demonstrated to ameliorate the clinical and histopathological severity of trinitrobenzene sulphonic acid-induced colitis. The therapeutic effect was associated with downregulation of the Th1-driven inflammatory responses and expansion of the Tregs compartment [99]. Similar findings were described in $MOG_{35–55}$-induced EAE. The therapeutic efficacy was maximal when MSC were administered at disease onset but no effect was documented after disease stabilisation [28]. In a similar model, MSC produced a substantial decrease in the number of inflammatory infiltrates, and reduced demyelination and axonal loss [29].

The prominent activity of MSC in preventing, rather than inducing, disease remission can be explained by two—not mutually exclusive if not complementary—hypotheses. One is that the effect on the disease is simply the result of MSC preventing the expansion of the pathogenic T cells [24]. In fact, in most of these models the disease is the consequence of an immunisation so that the MSC, rather than 'treating' the disease are inhibiting the conditions for this to occur. The other explanation involves the 'licensing' notion described previously. Since MSC require exposure to an inflammatory microenvironment to acquire their immunosuppressive properties, the environment that they encounter at the beginning (disease induction) and later (full blown disease) may have an opposite effect on the MSC therapeutic effects. Such a hypothesis can reconcile some of the discrepancies in the experimental data regarding the effect of MSC on collagen-induced arthritis [100, 101] and in graft-versus-host disease (GvHD). The studies on MSC in GvHD have produced important results to clarify the modalities of MSC therapeutics. Using an MHC-mismatched donor-recipient pair, it was observed that the infusion of MSC is not sufficient to prevent the development of GvHD [102]. Although not necessarily in conflict with these results, another study, using MSC of human umbilical cord blood origin, observed that the GvHD produced by human T cells in NOD/SCID mice could not be prevented when MSC were given right at the beginning of the experiment. In contrast, GvHD pathology and symptoms were fully prevented if MSC were given in multiple doses at weekly intervals although no effect was detectable if MSC were administered at the time of disease induction [103]. These apparently conflicting data were resolved when MSC were tested for their ability to prevent GvHD when given at different times in one single dose. In this study, it was observed that only when MSC were infused at day +2 or +20, did they significantly increase the survival of recipient mice [104]. At these times, the levels of IFN-γ were particularly high in this model and therefore likely to confer on MSC their immunosuppressive

activity. This clearly demonstrates the importance of MSC 'licensing' also in vivo [65]. Furthermore, other data suggest that the microenvironment can polarise MSC into the opposite direction: during the development of chronic rejection of transplanted hearts MSC shorten allograft survival [105].

In conclusion, the inflammatory environment to which MSC are exposed is a fundamental factor influencing MSC beneficial effects. It should also be noted that under some circumstances the lack of therapeutic effects could be ascribed to a weak activity of MSC on the underlying disease and/or to the fact that the disease, when established, is completely insensitive to the immunosuppressive activity.

10.12 MSC Mediated Immunomodulation for Therapeutics: The Clinical Experience

It is probably fair to say that in the MSC field clinical applications have preceded basic scientific investigations and, despite this being an unusual approach in biology, it has attracted significant attention and consequently large-scale experimental activity to understand and better exploit MSC properties.

The first convincing report that MSC could produce a potent immunosuppressive activity was the description of a young boy affected by severe steroid-resistant GvHD who failed all forms of second-line treatment. The patient received two doses of MSC intravenously which produced a durable remission [106]. More convincing was a later phase II multicentre clinical trial demonstrating that MSC administered to 55 patients with steroid-resistant acute GvHD produced complete remission in 30 patients and measurable improvement in a further 9 patients. Remissions were durable, since responders experienced a higher overall survival 2 years after HSCT than non-responders (53 vs 16 %) [64]. Although this conclusion sounds obvious, it is nevertheless incredibly important because aggressive immunotherapies can often improve acute GvHD but do not improve the survival rate. It should be noted that a previous multicentre study in which patients received MSC as a prophylaxis of GvHD failed to show any difference in the incidence or severity of GvHD [107]. Although apparently discrepant, these findings can again be explained by MSC-mediated immunosuppressive capabilities not being constitutive but inducible by inflammatory molecules. It is in fact plausible to surmise that MSC infused at the time of transplant did not encounter the full inflammatory—'licensing'—microenvironment generated later on during the GvHD process [65].

The clinical use of MSC in hematopoietic stem cell transplantation was reported even before the dramatic effects on GvHD. A total of 28 patients were given high-dose chemotherapy and autologous hematopoietic stem cells in conjunction with culture-expanded autologous MSC. MSC infusions at the time of transplantation produced a more rapid hematopoietic recovery as compared to historical controls [108]. Although the biological feature exploited in this case is not the immunosuppressive activity on an allogeneic response, it is nevertheless

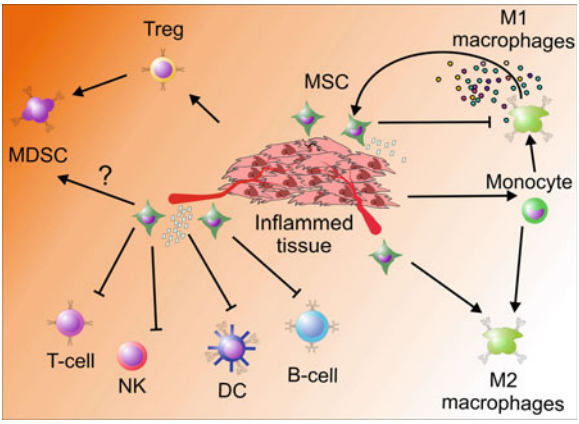

Fig. 10.1 Mesenchymal stem cells regulate inflammation as a result of a co-ordinated interaction with various key players. Following inflammation, pro-inflammatory macrophages (*M1*), by secreting molecules like IFN-γ, TNF-α, IL-1β, confer on MSC the ability to inhibit virtually all immune effectors, including T and B cells, natural killer (*NK*) cells and the antigen presenting function of monocytes and dendritic cells. Not only are monocytes/macrophages fundamental to 'license' MSC suppressive activities, they also play a crucial role in boosting MSC-regulatory properties. MSC polarise M1 into anti-inflammatory macrophages (*M2*) which also promote tissue repair. Considering the remarkable similarities, it is likely that myeloid-derived suppressor cells (*MDSC*) are also recruited by MSC. There is accumulating evidence that MSC activate and expand regulatory T cells (*Tregs*), thus further amplifying the immunosuppressive circuit.

likely that some of the anti-proliferative and anti-apoptotic mechanisms underlying such an activity [109] play a major role in facilitating the rebuilding of recipient hematopoiesis [10]. Recent studies confirm that the immunosuppressive and the graft facilitating effects can be effectively combined. The co-transplantation of donor MSC with HLA-disparate hematopoietic stem cells from a relative into a pediatric cohort of patients produced sustained hematopoietic engraftment as compared to a graft failure rate of 15 % in historic controls [110]. The results were similar in a cohort of adult patients [111].

Although it has long been proposed that the immunomodulatory properties may have a profound impact on solid organ transplantation, very few initiatives are currently being pursued, possibly because of the contrasting results that were summarised in the previous section. A very recent study in renal transplantation suggests that MSC could foster the donor-specific tolerogenic environment created by hematopoietic stem cell transplantation [112].

Patients with autoimmune diseases are also potential candidates for MSC therapies although the ethics of treating patients during the active phase of the disease are questionable. The use of MSC to control systemic inflammation in Crohn's disease could be promising but commercial studies have failed to provide supporting evidence. However, there are data indicating their effectiveness for the treatment of complex perianal fistula with high rates of healing [113]. Very interesting results have recently been reported with the use of allogeneic MSC in

four treatment-refractory systemic lupus erythematosus patients that showed a stable 12–18 months disease remission in all treated patients. Not only did the treatment ameliorate disease activity, but also produced improvement in serologic markers and renal function [114].

10.13 Concluding Remarks

Despite being poorly characterised, MSC have attracted much interest due to a number of properties ranging from their intrinsic ability to differentiate into mature cell types to their potent immunosuppressive activity. The data generated in the last few years have identified several mechanisms—partly redundant, partly synergistic—by which MSC inhibit cell proliferation, protect cells from apoptosis to soothe inflammation. When these activities target immune cells, they result in the temporary control of immune responses but the same effects can be exerted on parenchymal cells with the consequence of protecting them from cytotoxic insults, thus favouring spontaneous tissue repair. It is likely that this phenomenon is fundamental in physiological conditions in order to limit unnecessary inflammation and preserve tissue-specific stem cells. The fact that it is triggered by inflammation itself ('licensing') further supports this notion. The current therapeutic exploitation of these properties will clearly benefit from understanding the underlying mechanisms of in vivo 'licensing' and the respective profile of activities generated in the context of different disease microenvironments (Fig. 10.1).

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Part III
Induction of Immunological Tolerance

Chapter 11
The Immunogenicity of Stem Cells and Thymus-Based Strategies to Minimise Immune Rejection

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Abstract Stem cell research is advancing at a rapid pace, offering the possibility of personalised, "made to order" reparative stem cell treatments. A major challenge, however, is the immunological rejection of the transplanted tissue or 'allograft' that is not derived from self. Current clinical practice for overcoming graft rejection is to administer immunosuppressive drugs. Unfortunately these are associated with a number of side effects, including severe and often prolonged immune deficiency, which can lead to complications associated with opportunistic infections. Rather than prolonged global suppression of the immune system, strategies that focus on inducing graft-specific tolerance will provide a more robust and sustained approach to enabling successful translation of stem cell therapies to the clinic.

11.1 Introduction

It has been increasingly demonstrated in preclinical models that stem cells have the potential to cure a number of debilitating diseases [1]. However, the successful application of stem cell-derived therapies relies on the ability of the host immune system to accept the graft [2]. Unless the grafted tissue is derived from self, it will ultimately be rejected by the host immune system. Traditionally, strategies to overcome non-self or allogeneic graft rejection have been based on lifelong immunosuppression, which leads to high levels of morbidity stemming from opportunistic infections and malignancy.

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A logical solution to overcoming these problems is adaptation of the body's own mechanisms for inducing 'central tolerance' to self-antigens, allowing long-term acceptance of the graft, while maintaining immuno-competence. This can be achieved using donor hematopoietic stem cell (HSC) transplantation (HSCT) which, after bone marrow (BM) engraftment together with host HSCs and subsequent seeding of appropriate progenitor cells to the thymus, results in a mixed chimera of both self and donor hematopoietic and lymphoid cells [3]. Following this thymus-based "central tolerance" induction, the new T cell repertoire will be tolerant to both host and donor cell antigens. As successful as this has been in young animals, a major problem arises around puberty, when the thymus undergoes a natural, prolonged and ultimately profound decline in function with age. Although the direct mechanisms behind thymic atrophy have yet to be determined, a number of approaches have demonstrated that atrophy is at least partially reversible, with substantial restoration of thymic function [4]. Hence, strategies to generate central tolerance to stem cell grafts should be complemented with thymic regeneration.

11.2 Stem Cells

The idea that one cell holds the possibility to treat any number of diseases is fast becoming a reality with the advent of stem cell biology and research. A number of different stem cell types exist, including embryonic stem (ES) cells, adult (also including placental) stem cells and induced pluripotent stem (iPS) cells. Of these, ES cells are commonly considered the most pluripotent but iPS cells may well be equivalent; both thus hold great clinical potential, not only for treatment but also understanding disease processes. ES cells are derived from the inner cell mass of blastocyst-stage embryos and were, therefore, associated with a number of ethical and safety-related issues, although these have now been adequately addressed in most countries. ES cells cannot only self-renew indefinitely, but can differentiate into each of the three embryonic germ layers, endoderm, mesoderm and ectoderm. By the addition of specific growth factors, ES cells can be induced to differentiate into a wide variety of somatic tissues for potential therapeutic application [5, 6], including neurons and glia, cardiac muscle cells, blood progenitor cells, hepatocytes, retinal precursor cells, lung epithelial cells and β-cells of pancreatic islets [1]. They are characterised by the ability to spontaneously form differentiated structures known as embryoid bodies (EB) upon transfer onto non-adherent plates, or in vivo by their ability to form teratomas following injection into severe combined immunodeficient (SCID) mice. These teratoma structures are essentially tumours that contain a mixture of cell types from the three germ layers. In addition to immune rejection, it is this property, which precludes the ease of usage of ES cell-derived products clinically, as any contaminants may seed teratoma formation. This illustrates the critical requirement to remove any undifferentiated cells from

the ES or iPS cell-derived therapeutic cell population to be transplanted, to avoid the risk of tumour formation.

Unlike ES cells, adult stem cells are found throughout the body post-embryonic development. These stem cells are responsible for the homeostatic maintenance, repair and regeneration of the tissue or organ in which they reside. Some of the most characterised adult stem cells include HSCs of the BM, epithelial stem cells of the skin and satellite cells of skeletal muscle. However, despite their ability to self-renew and differentiate, they are restricted in their potential to give rise to other tissue types [7]. Some adult stem cells have been reported to possess ES cell-like characteristics and maintain the ability to give rise to all three germ layers in vitro. These include amnion epithelial cells, isolated from placental membranes [8]. Mesenchymal stromal cells (MSC) also exhibit multiple functions and differentiation potential. Initially isolated from the BM [9], adherent fibroblastic MSCs can expand in culture and give rise to bone, fat and cartilage in vitro. MSCs have since been isolated from various tissues including umbilical cord, placenta and adipose tissue [10]. MSCs also have characteristic anti-inflammatory/immunosuppressive properties, which are important for repressing inflammatory conditions and preventing immune rejection (at least in the short term) [11]. Despite being less "plastic" than ES cells, one important aspect of the use of adult stem cells for tissue regeneration is that they do not form teratomas upon transplantation, eliminating the safety issues associated with their embryonic counterparts [9]. There are now several clinical trials involving MSCs for a variety of clinical conditions including cardiac, osteopathic, hematopoietic and autoimmune diseases [12–15].

iPS cells are ES-like cells that were originally generated from terminally differentiated somatic cells, through the addition of pluripotency-associated genes. This revolutionary technology has now progressed dramatically, with the use of a wide variety of target cells (including adult stem cells) and more defined factors (including small molecules [16] and proteins [17]); without the original oncogenic transcription factors such as c-Myc [18]. iPS cells exhibit similar morphology, growth patterns and gene expression profiles to ES cells. Upon injection into SCID mice, iPS cells are also able to form teratomas [19]. These iPS cells hold the promise of overcoming immune rejection—a skin cells, for example, could be de-differentiated into an iPS cells, and then re-differentiated into a therapeutic cell population for transplantation into the patient. However, whilst, iPS cells may hold the same therapeutic potential as ES cells without being associated with ethical controversies, there remains safety issues associated with the profound genetic re-organisation and, in some systems, the use of viral vectors to deliver the relevant genes [19]. More recently, the potential for spontaneous dedifferentiation of the therapeutic cell population has become evident [19]. The former is now being overcome using proteins [16], mini-circle DNA [17] together with small molecules as delivery systems, rather than incorporation of genes permanently into the genome [20].

11.2.1 Stem Cell Transplantation

The therapeutic potential of stem cell transplantation is best exemplified by the use of bone marrow transplants (BMT) for over 40 years.[1] HSC are the most important cell type in BMT, because they not only replenish the hematopoietic compartment but they do so permanently. Both autologous and allogeneic HSCT are now well established and constitute a curative technique for many conditions including primary immunodeficiency disorders, autoimmune conditions, BM failure syndromes, non-malignant hematological disorders, as well as hematological malignancies [21]. Whereas autologous HSCT will not involve any rejection, a major problem with allogeneic HSCT is not so much the rejection of the foreign graft by the recipient's immune system (because they are immune suppressed from the conditioning), but the induction of graft-versus-host disease by T cells within the graft. This remains a major clinical problem, particularly since it needs to be delicately balanced with the beneficial effects of the graft versus leukaemia (GvL) effect mediated by the donor T cells [22].

11.2.2 The Immunogenicity of Stem Cells

Initially, there was a widely held belief that many types of stem cells evaded immune rejection because they themselves produced anti-inflammatory molecules or because they did not express sufficiently high levels of immune stimulating molecules. Indeed some studies have shown that undifferentiated ES cells can be transplanted across a minor histocompatibility (mH) barrier, seemingly without eliciting an immune response [23]. However, in most cases the transfer of cells, tissues or organs from one individual to another results in immunological rejection through host immune recognition of donor antigens.

There are three classes of "transplantation" antigens, namely major histocompatibility complex antigens (MHC), mH antigens (H) [24] and the blood group (ABO) antigens [25]. Of these, MHC mismatch is the major cause of allograft rejection. MHC proteins are classified as MHC class I, which is expressed on almost all nucleated cells, and MHC class II, which is expressed only on cells in the body that can present antigen. In humans, MHC proteins are known as human leukocyte associated antigens (HLA). There are three genes for each of the HLA classes, namely HLA-A, HLA-B and HLA-C for class I and HLA-DP, HLA-DQ and HLA-DR for class II. The HLA genes are the most polymorphic genes in the human genome, with some possessing several hundred alleles. Additionally, at least six different HLA alleles are expressed at any one time, in a co-dominant fashion [26].

[1] http://emedicine.medscape.com/article/1014514-overview

The probability of selecting a complete MHC match for any allogeneic transplanted tissue, including ES cells and their derivatives, is thus almost impossible. It has been suggested that creating an ES cell bank comprising 150 donors encompassing all ABO blood groups, or 100 universal blood group O donors, would include enough HLA haplotypes for matching the general population [27]. However, the success of this approach would be limited, since a single mismatch at any one locus can generate an immune response and ultimately induce rejection [23]. Moreover, ES cells and some stem cells derived from adult tissues express low levels of HLA class I, which is up-regulated upon differentiation into more mature cell lineages [28] or following exposure to pro-inflammatory cytokines [29]. Accordingly, there have been recent reports that ES cells are indeed immunogenic, eliciting readily detectable immune responses [30, 31].

T cells recognise alloantigen by two distinct pathways, direct recognition, whereby T cells recognise intact allogeneic MHC molecules, together with peptide on the surface of donor-derived dendritic cells (DCs) present in the graft. Indirect recognition involves the presentation of alloantigen by host derived antigen presenting cells (APC), that has been phagocytosed and processed for presentation by host-MHC [32]. Unlike tissue allografts, ES cell transplants do not contain DCs, professional APCs which express MHC class II. DCs are widely distributed throughout the body and, because of their role in priming the immune response via MHC class II antigen presentation to $CD4^+$ T cells, they play an important role in allograft recognition by the immune system. An absence of DCs from the ES cell graft eliminates direct antigen recognition [32]. Nevertheless, several studies have now indicated that ES cell-derived grafts undergo a progressive infiltration of inflammatory cells, which include neutrophils, macrophages, granulocytes, B cells and T cells [30, 31]. Furthermore, host-derived DCs and other APCs accumulate in the ES cell graft over time, leading to indirect graft recognition [30].

There is also evidence suggesting ES cell-grafts induce a humoral immune response. In support of this contention, high levels of allo-antibodies are found in transplanted mice, accompanied by high levels of T helper cell (Th)-2 cytokines, including interferon-γ and IL-4 [31]. Together this suggests that rejection of ES cell-grafts is mediated by both strong cellular and humoral immune responses.

A type of adult stem cell that appears to show some "immune privilege" is the MSC. Following transplantation, MSCs can home to sites of damage and produce cytokines and growth factors which suppress inflammation and induce tissue repair [33]. Early reports have shown that MSCs do not elicit overt allogeneic responses in vitro [34] suggesting that they will evoke little immunity when transplanted [11]. Hence MSCs have now been subjected to many clinical trials, highlighting their ability to restrain graft-versus-host disease, promote hematopoietic engraftment [10, 11] and repair bone fractures [14, 35]. In some cases, MSC induction of tissue repair may be by direct differentiation into the damaged cell type [36] or by indirect mechanisms, such as cytokine/growth factor production to reduce inflammation to enable tissue repair to progress [37]. Regardless, there is now accumulating evidence suggesting that allogeneic MSCs, like ES cells, are being recognised by host T cells, resulting in both cellular and humoral immune

responses [38–40]. This will need to be resolved, particularly if multiple dosing of MSCs from the same source is required for treating more chronic conditions.

11.2.3 Overcoming Immunological Barriers

In terms of avoiding transplantation rejection, iPS cells derived from the patient and Somatic Cell Nuclear Transfer (a technique where the nucleus of a donor somatic cell is removed and placed in an enucleated oocyte, creating a more primitive phenotype and plastic cell) [41] are promising alternatives. These techniques can be utilised to revert differentiated cells to a more pluripotent or even partially "stem cell" state, and then differentiate them into disease specific therapeutic cells, thus providing patients with a source of autologous tissue for transplantation. For example, disease-corrected iPS cells have been developed from patients with both hematological and neurodegenerative disorders [42]. However, many questions remain with regard to their clinical utility. As yet, it is not known if iPS cells will revert to their original diseased cell type, if they will form tumours and how they function in comparison to their natural counterparts.

The successful application of non-self stem cell-derived therapies relies on the ability of the host immune system to accept the graft. As previously mentioned, conventional strategies to overcome graft rejection are based on long-term, often lifelong, immunosuppressive regimes. While these can successfully aid graft acceptance in the short-term, they also lead to generalised immunodeficiency, precipitating opportunistic infections and even malignancy. A logical solution to overcoming these problems is to adapt the body's own highly successful mechanisms for inducing permanent tolerance to self-antigens to create a donor-specific tolerance, thereby facilitating long-term acceptance of the graft, while maintaining immune competence and minimising the use of immunosuppressive drugs. The organ responsible for "teaching" the body to distinguish self from non-self is the thymus.

11.3 The Thymus

The adult thymus is a 3-dimensional stromal network; composed of discrete cortical and medullary epithelial regions, mesenchyme-derived fibroblasts, BM-derived DCs and macrophages, as well as endothelial cells. Thymocytes interact with and migrate through these stromal cells as they differentiate into mature self-tolerant T cells before migrating to the periphery, to establish and maintain the T cell arm of immunity [43]. This thymic microenvironment provides a niche where specialised interactions between hematopoietic T cell progenitors and thymic stromal cells can occur, each contributing to the development and maintenance of the other, in a sophisticated course of events [44].

11.3.1 Thymus Development

In early embryogenesis the thymus originates from the endodermal layer of the anterior foregut. A homogenous population of epithelial cells, derived from the third pharyngeal pouch endoderm gives rise to the cortical and medullary regions of the thymus [45]. At this stage at least some of the thymic epithelial cells (TEC) are bi-potent, with the potential to differentiate into both cortical TECs (cTECs) and medullary TECs (mTECs) [46]. Expression of the Forkhead-box transcription factor N1 (FoxN1), restricted to epithelial cells, is initiated at approximately embryonic day (E) 11.5, a process essential for the downstream differentiation into cortical and medullary lineages and colonisation of the anlage by hematopoietic progenitors [47]. Following E11.5, FoxN1 is expressed by all epithelial cells in the rudiment [48] and maintained throughout thymus development, detectable in numerous TECs in the adult thymus [49]. The function of FoxN1 in the adult steady-state thymus is less well understood, but thought to be involved in the maintenance of the epithelium and homeostasis [50]. Wnt and bone morphogenetic protein (BMP) signalling are responsible for initiating [51] and maintaining FoxN1 expression [52].

The initial stages of TEC formation occur independently of thymocytes [53], while the later stages rely on specific interactions between the TECs and thymocytes [54]. Studies in mice have indicated that signals delivered by thymocytes are crucial for the maturation of cTEC and mTEC subsets from a common precursor, as well as the support and maintenance of the thymic architecture. In the mouse, thymic epithelial progenitor cells (TEPC) have been phenotypically identified as $MTS24^+$, keratin (K) 5^+ and $K8^+$ [55], with differentiation into mature cTEC subsets phenotypically marked by the loss of K5 and the retention of K8, whereas mTECs can be marked by the expression of K5 and the loss of K8 [53]. As development proceeds the thymus increases in size and compartmentalises into discrete specialised areas, which include an outer cortex housing the cTECs, and an inner medulla housing the mTECs, with the two regions being separated by the cortico-medullary junction [43].

11.3.2 Thymopoiesis and Central Tolerance

The thymus is responsible for providing T cells throughout adult life. To do so the thymus must recruit hematopoietic progenitors from the BM via the blood, a process known as thymus seeding (Fig. 11.1a). Thymopoiesis is initiated by thymic chemokines, attracting BM-derived progenitor cells expressing the receptors CCR7 and CCR9 [56], as well as recognition of P-selectin on thymic endothelium through P-selectin glycoprotein ligand (PSGL)-1 [57].

Upon entry, the blood-borne progenitor cells rapidly commence thymic commitment, following a number of well-defined differentiation steps, which occur

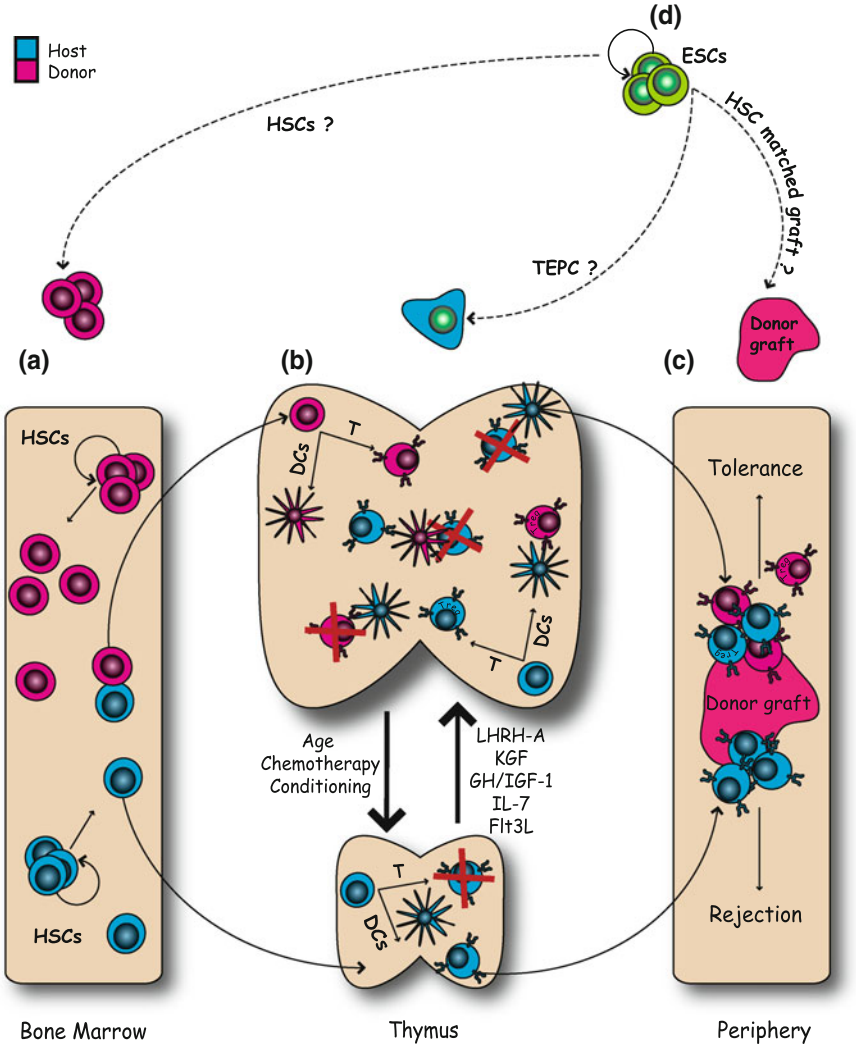

Fig. 11.1 Tolerance to an allogeneic graft can be induced by generating hematopoietic chimerism. Host cells are shown in *blue* and donor cells are shown in *pink*. **a** Following appropriate *BM* conditioning, transplanted donor *HSCs* engraft in the host *BM*, producing T cell and *DC* precursors, which migrate to the thymus. **b** In the thymus, precursors differentiate into T cells, *Tregs* and *DCs*. Reactive cells are deleted upon encounter with their cognate self-antigen presented by *TECs* as well as host and donor-derived *DCs*, a process known as negative selection. T cell output is significantly hindered in the atrophied thymus, which occurs through ageing, chemotherapy and *BM* conditioning. Thymic atrophy can be reversed through the ablation of sex steroids and/or the provision of growth factors. Educated T cells, along with both host and donor-derived *Tregs*, migrate to the periphery, **c** where tolerogenic host and donor-derived *Tregs* induce anergy in any reactive T cells that have escaped negative selection, promoting tolerance and graft acceptance. **d** Stem cell technologies may enable the generation of *HSCs*, *TEPCs* and tissue grafts from the same pluripotent stem cells for transplantation

within discrete thymic regions. Progenitors progress from immature $CD3^{-}CD4^{-}CD8^{-}$ (triple negative, TN) stage to $CD4^{+}CD8^{+}$ (double positive, DP) thymocytes, which, if able to recognise their respective peptide-MHC complexes with appropriate level of low affinity, receive a survival signal, in a process termed positive selection, to become mature $CD3^{+}CD4^{+}CD8^{-}$ or $CD3^{+}CD4^{-}CD8^{+}$ (single positive, SP) thymocytes. SP thymocytes are subject to a further developmental checkpoint whereby potentially auto-reactive cells binding with high affinity to MHC-peptide complexes are deleted from the repertoire (negative selection). The end result is a pool of naive T cells tolerant to self-antigens and capable of recognising a plethora of foreign antigens. This thymic, or "central" tolerance is mediated predominantly by DCs [58]. As dedicated antigen-presenting cells, DCs provide thymocytes with the optimal means of responding to self-peptides many of which they may encounter in the periphery [59]. Thymocytes expressing T cell receptors (TCRs) with high affinity for self-peptides presented by DCs undergo apoptosis [60], are functionally inactivated (anergy) [61], lose their auto-reactive TCR (editing) [62], or are directed into a mature T cell lineage such as regulatory T cells (agonist selection) [63]. In this way, potentially auto-reactive T cells are purged from the nascent repertoire.

It is now known that, in addition to DCs, mTECs play a major role in the induction of self-tolerance through their unique ability to promiscuously express a diverse range of genes usually constrained to peripheral tissues, such as insulin, thyroglobulin, myelin oligodendrocyte glycoprotein and the acetylcholine receptor [64]. The expression of these peripheral tissue antigens (PTAs) by mTECs is under the control of the autoimmune regulator (AIRE), which is therefore crucial for preventing the development of autoimmunity [65].

11.3.3 T Regulatory Cells

Although highly efficient, central tolerance is not foolproof. Consequently, other peripheral mechanisms are present, ensuring auto-reactive T cells that have escaped negative selection, do not cause autoimmunity. $CD4^{+}CD25^{+}$ FoxP3-expressing T regulatory cells (Tregs) play an essential role in maintaining self tolerance and preventing T cell mediated autoimmune diseases [66]. "Natural" Tregs, like other T cells, are generated in the thymus through encounter with TCR-agonist ligands expressed on thymic epithelium with an intermediate level of activation signalling [67]. However, unlike conventional $CD4^{+}$ and $CD8^{+}$ T cells, the majority of Tregs are specific for self [68] and are continuously activated [69]. Following thymic emigration, Tregs, along with naïve T cells, home to draining lymph nodes. Here encounter with antigen leads to Treg activation, inducing proliferation and enhancing suppressor function [70]. Activated Tregs regulate neighbouring $CD4^{+}$ and $CD8^{+}$ T cell responses, via cytokine production, and cell-to-cell contact [71], preventing their proliferation [70] and ability to function as effector cells [72].

Thymic selection is not the only means of producing Tregs, since circulating naïve $CD4^+$ T cells can be selected to form so-called "induced" $CD4^+CD25^+$ Treg cells in the periphery via encounter with organ-specific agonist ligands [73]. Furthermore, such Tregs can be intentionally generated by the application of specific agonists under sub-immunogenic conditions (low dose and/or lack of co-stimulation) [74].

11.4 Manipulating the Thymus for Transplantation Tolerance

Given the thymus relies on continual seeding by BM precursors [75], it is possible to supply donor-derived hematopoietic progenitors and induce a state of mixed chimerism; the co-existence of donor and host hematopoietic cells in the same tissue (Fig. 11.1c). Advances in stem cell technologies may one day enable the generation of both HSCs and tissue graft from the same pluripotent stem cell (Fig. 11.1d). If so, the thymus provides all the necessary attributes to "teach" the body to accept the donor graft, essentially re-programming the immune system for the life of the recipient.

11.4.1 Chimerism

Mixed chimerism has been used successfully in many rodent models for the specific induction of allogeneic tolerance. Owen first observed this process over 60 years ago, demonstrating that a mixture of two distinct types of erythrocytes can be found long after birth in fraternal bovine twins that had shared a common placental circulation [76]. Importantly, this was induced in the neonatal period. Shortly afterward, Medawar and colleagues demonstrated that skin grafts between these bovine chimeric twins were accepted indefinitely, indicating that each had acquired a tolerance to the other's tissue [77]. Ten years later, Billingham and colleagues showed that this form of tolerance could be actively induced between MHC-disparate mice, provided the skin graft recipient mice had been exposed to donor antigen in the neonatal stages of development [78].

The principle is simple and can be applied to adults with appropriate conditions, providing successful HSC engraftment and the existence of an active thymus. Recipients are given a BMT or HSCT from an allogeneic donor that is MHC-matched to the tissue (such as skin) to be transplanted. These cells engraft in the BM and differentiate into lymphoid and myeloid progenitors. T cells and DC precursors from both host and donor migrate to the thymus whereby the process of negative selection purges the emerging T cell pool of host-reactive as well as donor-reactive T cells [79] (Fig. 11.1b). When the appropriate immunosuppressive regimes have been applied to eliminate pre-existing mature allo-reactive T cells in

the periphery, tolerance to fully MHC-mismatched skin grafts can be consistently achieved [80]. This has also been observed in clinical settings where patients who had received BM transplants for haematological malignancies subsequently became tolerant to skin [81] and kidney [82] grafts originating from the same donor, with the additional contribution of newly produced thymus derived donor-specific Tregs [83].

Donor-specific Tregs are involved in the induction and maintenance of allogeneic tolerance, through their ability to promote donor cell engraftment [84] and prevent both allogeneic graft rejection [85] and graft versus host disease (GvHD) [86, 87], indicating that they play an important role in the long-term survival of the graft, by providing a natural, more-specific means of immunosuppression [88]. Furthermore, Tregs have recently been shown to play a crucial role in preventing the rejection of allogeneic ES cell-derived grafts, indicating a possible mechanism behind the initial idea that ES cells were "immune privileged" [89].

To allow donor BM cells or HSCs to engraft within the recipient, existing mature alloreactive T cells must be eliminated and "space" must be created within the recipient BM, in a process referred to as "conditioning". To induce a permanent state of immunological tolerance, cells that have engrafted in the BM must have a lifelong multilineage repopulating ability in order to provide the thymus with a constant source of donor antigens. The simplest and most reliable method for creating "space" in the BM is total body irradiation (TBI) prior to transplantation of T cell-depleted donor marrow. The dose is both myeloablative, to create "space" for donor cells, and immunosuppressive, to eliminate the potential for developing host-versus-graft disease (HvGD). Using this approach successfully should induce fully MHC-mismatched allogeneic and xenogeneic (across species) graft tolerance [90].

11.4.2 Thymic Atrophy

A problem that is frequently overlooked with the above approach, is that the thymus, the principal organ responsible for generating a pool of T cells tolerant to both donor and host tissue, undergoes a profound atrophy with age (Fig. 11.1b). Thymic function is most active during the fetal and perinatal stages of development, with a decline in function evident from as early as the first year of human life. Thymic degeneration is progressive and most apparent at puberty, with approximately 95 % of thymus function lost by 50 years of age [91].

This age-related thymic involution is characterised by gradual changes in the thymic microenvironment, including a loss of distinction between cortical and medullary regions, extensive vacuolisation of epithelial cells, and the replacement of thymopoietin tissue with perivascular spaces and adipose deposits [91]. Detailed analysis of thymic stromal subsets reveals an increase in proportion of non-TECs such as fibroblasts, while a decrease in TEC number and proportion of particularly mTECs is evident within the TEC compartment [92]. In addition to thymic stromal

changes, there is a decline in early T cell progenitors, which also display a reduced capacity for differentiation [93].

Together these processes result in decreased production and export of naïve T cells from the thymus, leaving homeostatic proliferation of T cells in the periphery to compensate for this loss. As T cell maintenance in the elderly relies on the expansion of mature T cell subsets rather than naïve T cell emigrants, the diversity of the T cell pool undergoes a bias towards antigens that have already been encountered by the immune system. Within this constricted TCR repertoire, the likelihood of matching the appropriate TCR to novel antigenic epitopes decreases, ultimately limiting the immune system's ability to recognise and respond to unfamiliar challenges [94].

The mechanisms behind thymic atrophy are not clear and several factors have been implicated (Fig. 11.1b). Of these, sex steroid production has been the subject of numerous studies as puberty coincides with the greatest period of thymic involution [91]. Studies have also suggested reduced production of immunostimulatory growth factors and cytokines such as growth hormone (GH) [95] and interleukin (IL) 7 [96], as well as down-regulation of adhesion molecules required to facilitate thymocyte entry to the thymus [97]. Conversely, up-regulation of atrophic factors such as transforming growth factor (TGF) β may also contribute to the involution process. BM progenitors undergo a reduced lymphoid potential and self-renewal capacity with age [98]: since the thymus relies on the BM for continual seeding, age-related BM dysfunction may also play a role in the loss of thymic function with age.

11.5 Thymic Regeneration

One of the fundamental requirements for the induction of tolerance is a functional thymus, which can produce naïve T cells. Age-related thymic atrophy therefore presents a significant challenge for the development of chimerism-based approaches to the induction of tolerance to stem cell grafts in adult patients. Any strategies that manipulate central tolerance for transplantation therapies in the adult should, therefore, be coupled to the restoration of thymic function. Several preclinical and clinical approaches to restore thymic function have been proposed, including hormone blocking therapies and administration of growth factors to regenerate the ageing thymus (Fig. 11.1b). Furthermore, the identification of a putative TEPC may aid the de novo generation of thymic tissue.

11.5.1 Thymic Epithelial Progenitor Cells

The existence of a putative TEPC was demonstrated in the mouse embryo when $MTS24^+$ TECs engrafted under the kidney capsule of nude mice produced both mTECs and cTECs and gave rise to a fully functioning thymus capable of

supporting T cell development [55]. These MTS24$^+$ cells are abundant in the embryo, but become increasingly less frequent as the thymus develops, localising to the medulla and cortico-medullary junction in the adult mouse thymus. These progenitors also co-express both mTEC and cTEC markers K5 and K8 [55], supporting the hypothesis that the thymus develops from a bipotent TEC progenitor. More recent work has demonstrated that the MTS24$^-$ TEC population was also able to give rise to an ectopic thymus graft, but only when a significantly higher number of cells were reaggregated [99]. In the adult thymus, the existence of a TEPC is supported by the ability of both mTEC and cTEC to regenerate after both injury-induced and age-related thymic damage [100]. However, an adult TEPC phenotype has yet to be elucidated.

While identification of a resident adult TEPC should allow in situ manipulation of the thymus to enhance regeneration, it may be possible to generate a TEPC ex vivo from ES cells or even iPS cells. Factors that direct the differentiation of ES cells into the endodermal lineage are still relatively novel [1] and protocols to guide the development of thymus-specific tissue have yet to be established. Candidate pathways include the Wnt and BMP signalling families as both play a role in regulation of the FoxN1 transcription factor required for both TEC formation and maintenance [101]. Once established, ES cell-derived TEPCs could be directly injected into the atrophic thymus or grafted as reaggregate cultures under the kidney capsule, re-establishing function [55].

11.5.2 Sex Steroid Ablation

Evidence for residual thymic function, albeit very limited, in ageing individuals gives credence to the possibility of inducing thymus regeneration in vivo via the removal of inhibitory factors or administration of stimulatory factors. Of the former, sex steroids have been strongly linked to thymic atrophy, since they have considerable inhibitory effects on both lymphoid development and immune function [91]. Sex steroids exert direct effects on the thymic stromal cells, which express sex steroid receptors on the cell surface [102]. Consequently, removal of sex steroids through castration (chemical or surgical) is associated with marked rejuvenation of the thymic compartment in aged mice and following chemically induced thymic damage [100]. This is evident in both thymic architecture [92] and thymic cellularity [100, 103]. Specifically, regeneration is demonstrated by the restoration of cortical and medullary regions, TEC and fibroblast ratios, as well as TEC and thymocyte numbers. Importantly, thymic export of naïve T cells is increased, resulting in enhanced cytolytic activity upon viral infection [104].

Additionally, these improvements correlate with an increase in BM lymphopoiesis, in particular, an increase in the number of IL-7-responsive progenitor cells, an increase in B cell export, as well as enhanced B cell function [105], therefore contributing to an overall improvement in immune competence [106, 107]. The clinical relevance of sex steroid ablation is further demonstrated by accelerated

recovery from chemotherapy and irradiation-induced damage [102, 103, 108]. Of particular relevance to donor-derived tolerance induction, the removal of sex steroids has also been shown to improve engraftment in the BM and peripheral reconstitution following allogeneic BMT, without exacerbating GvHD [107]. If sex steroid ablation can increase thymic seeding by donor progenitor cells and thymic output of donor-tolerant naïve T cells, this strategy could complement BMT protocols for the generation of hematopoietic chimerism for tolerance.

Sex steroid inhibition can be achieved in a reversible manner with the use of a luteinising hormone releasing hormone (LHRH) analogues. LHRH is normally produced by the hypothalamus to stimulate pituitary secretion of luteinising hormone and follicle stimulating hormone, which in turn trigger sex steroid production by the gonads. LHRH agonists (LHRH-A) cause sensitisation and down-regulation of LHRH receptors, resulting in the shutdown of sex steroid production [97]. This process is reversed upon cessation of treatment. Clinical application of sex steroid inhibition in the context of immune regeneration has been demonstrated by improved thymic and immune recovery of LHRH-A-treated patients from autologous or allogeneic HSCT for hematological malignancies [104].

11.5.3 Keratinocyte Growth Factor

Keratinocyte growth factor (KGF) is a fibroblast growth factor that stimulates the proliferation of epithelial cells in a number of tissues. In the thymus, KGF is produced by mesenchymal cells and mature SP thymocytes and plays a significant role in regulating thymic epithelium development and function [109]. Although a deficiency in KGF does not accelerate thymic involution, mice deficient in KGF are unable to reconstitute the peripheral T cell compartment following BMT [110]. By stimulating TEC proliferation [111], exogenous KGF protects these cells from damage induced by cytoablative conditioning [110] and GvHD [112]. In mice receiving allogeneic BMT, KGF treatment enhances recovery of thymic cellularity, thymic function and peripheral T cell reconstitution [110]. Furthermore, KGF supports immune recovery in an additive manner when used in combination with LHRH-A [113].

11.5.4 Growth Hormone

Another key factor that has been associated with thymic involution is GH. GH is known to stimulate thymopoeisis and regulate a number of immunological events in the periphery [114]. Serum levels of GH, as well as expression of gherlin, a GH secretagogue, and its receptor in thymic stromal cells, decrease progressively with age [115]. In old mice, ghrelin infusion improves thymic architecture and increases

T cell output and diversity [116]. Similarly, high-dose GH treatment of HIV-infected patients has been shown to increase thymic export and naïve $CD4^+$ T cell numbers [117]. The effects of GH treatment are not restricted to the thymus as recombinant GH can also reverse irradiation-induced loss of BM progenitor function in mice [118].

GH mediates its immunostimulatory effects primarily through local insulin-like growth factor (IGF)-1 [119], expression of which also decreases with age [120]. In the context of immune regeneration, IGF-1 administration to mice receiving allogeneic BMT has been shown to support both lymphoid and myeloid reconstitution, without exacerbating the development of GvHD [121]. Recently, IGF-1 has been shown to exert its effects in a tissue-specific manner, with neutralisation of local IGF-1 to specific BM stem cell niches reversing the age-related decline in progenitor function [122]. Clinically, however, GH has many side effects including increased susceptibility to diabetes.

11.5.5 IL-7

IL-7 is a growth factor essential for T and B cell development [123]. In the thymus, IL-7 is produced by TECs and decreased IL-7 production with age has been associated with a reduction in IL-7^+ TECs [124]. While it remains unclear whether the decline in IL-7 production is the causative factor in thymic atrophy, IL-7 treatment has been shown to reverse involution-associated changes [125] and enhance peripheral T cell reconstitution in mice after BMT [126, 127]. Interestingly, combination therapy with IL-7 and IGF-1 has an additive effect on B cell but not T cell reconstitution in mice receiving allogeneic BMT [121]. In contrast, concomitant use of IL-7 and sex steroid ablation exerts profound additive effects in the thymus after allogeneic BMT [107]. Importantly, IL-7-treated patients with refractory cancer exhibit preferential expansion of naïve T cells and a more diverse T cell repertoire [128].

11.5.6 Flt3L

Fms-like tyrosine kinase 3 ligand (Flt3L) is another growth factor that can support thymic function and immune reconstitution. Flt3L is recognised by cells that express Fms-like tyrosine kinase 3 (Flt3) which include BM progenitors and immature thymocytes [129]. Unlike IL-7 that primarily acts to enhance peripheral T cell expansion, Flt3L exerts its effects by promoting BM progenitor expansion and downstream thymopoiesis and peripheral T cell reconstitution [129]. In the steady-state thymus, interactions between $Flt3^+$ T cell progenitors and thymic fibroblasts expressing Flt3L are important for maintaining T cell development [130]. Importantly, thymocyte recovery from irradiation-induced damage appears to require Flt3 ligand-receptor interactions [131].

11.6 Conclusion

Given that it is now clear that stem cells are immunogenic (even if some also have immunosuppressive or anti-inflammatory properties), the challenge of overcoming immunological rejection must be addressed before "made-to-order" stem cell transplantation can become a reality. Current methods focussing on long-term immunosuppression are associated with many adverse side effects and, in some circumstances, can, ironically, ultimately lead to graft rejection triggered through infection. Newer approaches which utilise graft-matched HSCT, manipulate the body's own mechanisms to induce tolerance, relying on the thymus to teach the immune system to accept the graft and produce graft specific Tregs for peripheral tolerance. This should provide long-term, low morbidity graft acceptance. Without a functionally active thymus, however, this process becomes severely limited. Hence, to achieve lasting donor-specific immunological tolerance, donor HSCT should be coupled to thymus regeneration.

This goal is clinically feasible, considering a number of therapies that can potentially restore thymic function already have Food and Drug Administration (FDA) approval for use in the clinic, albeit for other conditions. LHRH-A has been used for many years now to treat prostate cancer, endometriosis, fibroids and precocious puberty. KGF has recently been approved for the prevention of chemotherapy-induced mucositis in patients undergoing HSCT. While GH is used routinely to treat conditions caused by GH deficiency, it has a short half-life and supraphysiological doses are often required to achieve efficacy. This raises concerns associated with side effects and toxicity of GH. Hence, a safer alternative to improve immune recovery following HSCT, enhance uptake of donor HSC and subsequent development of donor antigen tolerance, may be temporary sex steroid blockade in combination with KGF or IL-7. These options may present the ideal platform for inducing long-lasting tolerance to stem cell-derived therapies.

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Chapter 12
The Induction of Mixed Chimerism Using ES Cell-Derived Hematopoietic Stem Cells

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Abstract The use of hematopoietic stem cell (HSC) transplantation for the establishment of mixed chimerism generally leads to durable immune tolerance to allografts in animal models of transplantation. The development of reduced intensity regimens for achieving allogeneic hematopoietic engraftment across major histocompatibility complex (MHC) barriers and its recent application in clinical trials of kidney transplantation is encouraging. Embryonic stem cell-derived HSCs have lower immunogenicity and could, therefore, potentially be safer, inducing mixed chimerism and tolerance with minimal host pre-treatment and risk of graft versus host disease, despite crossing MHC barriers.

12.1 Introduction

The use of hematopoietic stem cell transplantation (HSCT) for the establishment of mixed chimerism represents a viable and attractive approach for generating tolerance in transplantation biology, as it generally leads to durable immune tolerance, enabling the subsequent engraftment of organ transplants without the need for a deleterious, continuous immunosuppressive therapy. Currently, human bone marrow (BM), mobilized peripheral blood, and umbilical cord blood (UCB) represent the major sources of transplantable hematopoietic stem cells (HSCs), but their availability for use is restricted by both limited quantity and histocompatibility.

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Attempts to maintain or expand human HSCs in culture for even relatively short periods of time have been unsuccessful due to terminal differentiation. Successful combination of HSCT with solid organ transplant requires minimizing transplant-related mortality by eliminating the risk of graft-versus-host disease (GvHD) and by reducing the toxicity of the conditioning regimen used to establish long-term mixed chimerism. To avoid these severe side effects, new data on embryonic stem cells (ES cells) have sparked hope that mixed chimerism and tolerance with minimal host-pretreatment and minimal risk of GvHD can be achieved. In addition to the high degree of pluripotency, ES cells can generate newly differentiated cells that appear to have low immunogenicity, an ideal property for allogeneic transplantation. ES cells may also serve as a source of composite tissue grafts of HSCs, dendritic cells, mesenchymal stem cells (MSCs), and/or T regulatory (Treg) cells. Using ES cell-derived cells to achieve mixed chimerism may, therefore, provide the opportunity to accomplish immunological tolerance without the need for harsh host pre-conditioning and without GvHD despite crossing major histocompatibility complex (MHC) barriers.

12.2 Historical Perspective

In the 1940s Owen provided the first finding that tolerance to allografts was strongly associated with donor leukocyte chimerism, observing that acceptance of even fully MHC-mismatched skin is possible between particular cattle twins, the so-called ‘freemartin cattle’ that are naturally occurring chimeras, because of a common placental circulation [1]. Almost 15 years later, Billingham actively induced tolerance by injecting allogeneic cells into neonates, leading to a chimeric state associated with specific tolerance toward skin grafts from MHC disparate donor mice [2].

Based on these observations, hematopoietic chimerism was experimentally induced in adult mice with lethal doses of total body irradiation (TBI) causing global destruction (myeloablation) of the hematopoietic repertoire in the host which was then completely reconstituted with donor bone marrow cells (full chimerism), achieving acceptance of a donor skin homograft [3]. However, as mentioned, complete destruction of the hematopoietic compartment to facilitate 100 % donor engraftment is quite toxic, both in terms of the regimen side effects on other organ compartments and the risk of severe GvHD. Subsequently, the focus shifted to establishing mixed chimerism when it was discovered that irradiated mice reconstituted with a mixture of T cell depleted host and donor bone marrow developed mixed chimerism, accepted fully MHC-mismatched donor skin grafts permanently, rejected third-party grafts, and did not develop GvHD [4, 5].

12.3 Mixed Chimerism

All successful protocols for the induction of mixed chimerism in the preclinical and clinical settings involve three major elements: (1) non-myeloablative but myelosuppressive treatment to create space in the marrow niche for donor hematopoietic engraftment; (2) an immunosuppressive treatment (often involving T cell depletion) to prevent rejection and GvHD; and (3) a source of allogeneic HSCs.

12.3.1 The Rationale for Mixed Chimerism Induced Tolerance

Immune tolerance to a set of antigens can be defined as a state in which the immune system does not mount a destructive response to organs or tissues expressing those antigens, but is capable of responding normally to other foreign or third-party antigens. In the laboratory, tolerance is defined as antigen-specific non-responsiveness. In a clinical context, tolerance can be defined 'operationally' when an allograft is accepted long-term without the need for continuous immunosuppressive therapy.

Although several other experimental approaches have been suggested to induce tolerance, such as donor-specific transfusion or costimulation blockade, to date, proof of concept has been demonstrated in humans only by means of HSCT-induced chimerism [6, 7]. By definition full chimerism is 100 % donor hematopoiesis; mixed chimerism is the simultaneous co-existence of both donor and recipient hematopoiesis; microchimerism is persistence of donor hematopoietic cells at very low levels.

As mentioned, full chimerism is associated with a higher risk of severe GvHD, somewhat reduced immunocompetence, and increased short- and long-term toxicity from marrow ablative regimens compared to 'mixed chimerism' [8, 9]. Therefore, the focus in organ transplantation has generally been directed to establishment of stable mixed chimerism. In general, upon induction of mixed hematopoietic chimerism, which describes a balance of donor and recipient cells coexisting in the host, the recipient thymus, through the process of negative selection, mediates deletion of host and donor-reactive T cell clones as long as chimerism persists, ideally lifelong [10–12]. Intrathymic (or central) clonal deletion provides a very robust form of tolerance in all chimerism approaches achieving acceptance of even the most immunogenic tissues, such as skin and small intestine [5, 13]. Central deletion is usually regarded as superior to peripheral regulatory or anergic mechanisms since clonal deletion physically eliminates T cells with detrimental host or donor specificity. In peripheral tolerance (regulatory and/or anergic), cells remain present in a nonreactive state within the individual, but environmental influences may break tolerance. Mixed chimerism also provides antigen-presenting cells (APCs) of both donor and recipient in the periphery, preserving immunocompetence to antigen presented in peripheral tissues.

12.3.2 Murine Models of Mixed Chimerism

Alternatives to harsh myeloablative therapies were pursued in mouse experimental models wherein the concomitant infusion of high-dose bone marrow with less intense non-myeloablative regimens established mixed chimerism and promoted the deletion of donor-reactive cells in the thymus [10, 14]. T cell depletion in combination with thymic irradiation permitted the induction of chimerism and tolerance without TBI if very high doses of BM were transplanted. T cell-depleting antibodies, eliminating the main players of acute rejection and GvHD, were important in development of non-myeloablative protocols [4, 11, 15–17]. Anti-CD4 and anti-CD8-depleting monoclonal antibodies (mAbs), plus a more restricted irradiation to the thymic region, permitted lower doses of TBI [4]. Thymic irradiation could be eliminated by repeated administration of T cell-depleting antibodies [11]. However, irradiation could not be eliminated altogether, even with very high doses of T cell-depleting mAbs [16].

12.3.3 Costimulation Blockade-Based Mixed Chimerism in Murine Models

Costimulatory blockade (especially the CD28 and CD40 pathways) has been reported to eliminate the need for cytoreduction and provide long-term graft survival across multiple organ systems in experimental models [12, 13, 18, 19]. Anti-CD40L (CD154) mAb and the fusion protein CTLA4Ig currently provide the least toxic way of preventing alloreactive T cell responses in mixed chimerism regimens [18–20]. Importantly, when given alone, costimulation blockade does not induce true tolerance as demonstrated by rejection of skin grafts [21, 22], the most stringent test for tolerance, but they can be very efficiently used as part of BM transplantation (BMT) protocols for tolerance induction through mixed chimerism [19, 20, 23].

Recently developed BMT models are mainly based on costimulation blockade and non-myeloablative doses of TBI or busulfan. Various dosing and timing regimens have led to an ever increasing number of BMT protocols. Protocols differ in the administration of costimulation blockade. Anti-CD40L is often used in combination with CTLA4Ig, but anti-CD40L alone is used in other mixed chimerism protocols [18, 19, 24–28]. A wide range of doses of costimulation blockade has been used from 0.5 to 8 mg/mouse in single or multiple injections. Moreover, different strain combinations are used for tolerance induction. Some protocols involve MHC-mismatched animals on the same genetic background (i.e. MHC-congenic combinations, such as B10.A to B6) [19, 24], while others employ MHC mismatches plus mismatches at minor histocompatibility (mH) antigens (for example Balb/c to B6) [18, 27–30].

TBI remains an essential component in most protocols but instead of TBI, non-myeloablative doses of busulfan have been used in addition to costimulation blockade [18, 31]. Alternatively to high-dose BMT protocols, the minimally required dose of TBI can be reduced by short-term treatment with certain conventional immunosuppressive agents. Since only transient, their administration would be clinically acceptable provided that true tolerance can be achieved thereafter. A 4-week course of rapamycin-based immunosuppression allows a reduction in the dose of TBI from 3 gray (Gy) down to 1 Gy in a costimulation blockade-based model [29], plus it helps to reduce the minimally required dose of BMC required for BMT without any TBI [32]. It would be clinically desirable to completely avoid TBI due to its toxic effects, especially risk of late radiation-related cancers. Proof-of-principle studies have shown that mixed chimerism and tolerance can indeed be induced without any irradiation when transplanting approximately 10-fold higher doses of allogeneic bone marrow under the cover of costimulation blockade [12, 28, 33, 34].

12.3.4 Mixed Chimerism Using Composite Tissue Hematopoietic Allografts

Mixed chimerism has been shown to lead to prolonged MHC-disparate allograft survival and immune-specific tolerance. Studies found that cells other than HSC in the graft, such as passenger leukocytes or dendritic cells, play a critical role in microchimerism [35]. The interaction between leukocytes from the transplanted allograft and the recipient's own leukocytes may lead to the induction of donor-specific tolerance. Therefore, composite tissue grafts composed of HSC and other defined hematopoietic lineage cells are being evaluated for allograft tolerance. Yu et al. [36] reported that successful mismatched bone marrow engraftment can be achieved using immature dendritic cells (imDCs) given up to 3 days prior to BMT leading to stable chimeras and allowing the long-term survival (>110 days) of mismatched cardiac and skin allografts without evidence of GvHD and without the need for immunosuppression or myeloablation. Immature DCs can suppress lymphocyte proliferation in response to mismatched MHC stimulation, leading to increased expression of IL-4, IL-10, and decreased expression of IL-2 and interferon-γ [36].

MSCs are rare residents of the bone marrow compartments but may be involved in the induction of hematopoietic chimerism and subsequent immune tolerance during BMT. Pan et al. [37] found that fourteen of the fifteen recipients, to which MSCs were co-administered, developed stable and high level mixed hematopoietic chimerism whereas only two of the seven recipients not infused with MSCs developed stable chimerism. Most importantly, they found that no GvHD was observed in any of the recipients infused with a composite tissue graft containing MSCs [37].

Numerous studies show that regulatory T cells (Tregs) play a critical role in maintaining self-tolerance. The therapeutic exploitation of Tregs is beneficial in many experimental contexts, including autoimmune, organ transplantation, and GvHD models. Pilat et al. [38] used a protocol by which polyclonal recipient Tregs were co-transplanted with a moderate dose of fully mismatched allogeneic donor BM into recipients conditioned solely with short-course costimulation blockade and rapamycin. This combination treatment led to long-term multilineage chimerism and donor-specific skin graft tolerance [38]. A comparable magnitude of chimerism had previously been obtained with ten times as many BM cells transplanted with costimulation blockade alone [12, 28]. Both deletional and nondeletional mechanisms are involved in maintenance of tolerance, apparently not requiring long-term persistence of the transferred Tregs [38].

12.3.5 Mixed Chimerism Without Use of Chemo-Radiotherapy Conditioning Regimens

Wide-spread clinical application of hematopoietic mixed chimerism to induce solid organ tolerance depends on development of minimally toxic regimens for allogeneic HSC engraftment. The current conditioning regimens contain chemo-radiotherapy to "make room" in the host marrow niche for donor HSC engraftment as well as chemo-radiotherapy and antibodies against recipient immune cells to prevent allogeneic HSC rejection. In an immune deficient mouse, use of an antibody against a HSC-specific antigen (c-kit) allowed high-level allogeneic HSC engraftment without chemo-radiotherapy [39]. Although untested, it is possible that in an immune competent recipient the combination of an anti-stem cell antibody to make room in the marrow niche for allogeneic HSCs engraftment combined with antibodies to various components of the recipient's immune system to prevent recipient-mediated donor rejection—and against infused donor immune cells to prevent donor-mediated GvHD—may allow allogeneic engraftment and mixed chimerism without use of any chemo-radiotherapy.

12.3.6 Mixed Chimerism in Large Animals and Nonhuman Primates

In contrast to murine protocols that induce high levels of chimerism and robust tolerance with mild BMT regimens, large animals, and nonhuman primates require more extensive conditioning regimens (Table 12.1). Based on their previous mouse data, Strober et al. [34] established protocols for tolerance induction in dogs that involved total lymphoid irradiation (TLI) and T cell depletion with anti-thymocyte globulin (ATG) combined with donor BMT. Compared to the rodent

Table 12.1 Large animal and nonhuman primate models of hematopoietic stem cell transplant induced solid organ tolerance

Animal	Regimen utilized to facilitate HSC engraftment	Source of hematopoietic cells (BM, PBSC, purified, mega-dose)	% Chimerism—full or mixed	Organ transplanted	Long-term allogeneic organ tolerance without immune suppression
Dog [34]	TBI plus ATG	BMT (0.5×10^9 cells/kg)	N/A	Heart	Dog#1 > 360 days; Dog#2 > 494 days; and Dog#3 > 495 days
Monkey [45]	TBI, TI and ATG	BMT	N/A	Kidney	4–70 months
Monkey [47]	TBI, TI and ATG	BM mononuclear cells ($0.4–4 \times 10^8$ cells/kg)	N/A	Heart	138–509 days
Swine [44]	TI	CM-PBMC ($1–2 \times 10^{10}$ cells/kg)	N/A	Kidney	26/35 mixed chimeric recipients tolerant without immune suppression for 200–600 days
Canine [42]	TBI	BM nucleated cells ($3.3–7.5 \times 10^8$ cells/kg)	N/A	Kidney	5/5 mixed chimeric recipients tolerant without immune suppression for 8–17 months
Monkey [46]	TBI, TI and ATG	BM mononuclear cells ($0.3–3.5 \times 10^8$ cells/kg)	N/A	Kidney	8/13 mixed chimeric recipients tolerant without immune suppression for 137–3,478 days
Dog [140]	TBI	BM mononuclear cells ($2.07–3.34 \times 10^8$ cells/kg)	2.5 and 97.5 % mixture of donor and host cells	Renal and skin	Alive, 2,078 days post-op, normal renal function
Swine [141]	TBI and T cell depletion	Donor hematopoietic cells infusion	N/A	Skin	2/4 mixed chimeric recipients tolerant without immune suppression for 46–300 days

ATG anti-thymocyte globulin, *BM* bone marrow, *BMT* bone marrow transplantation, *CM-PBMC* cytokine-mobilized peripheral blood mononuclear cells, *N/A* no applicable, *TBI* total body irradiation, *TI* thymic irradiation

experience, the TLI regimen had to be decreased due to toxicity in large animals; these necessary changes in the TLI regimen decreased its efficacy (0 of 12 dogs achieved long-term heart allograft acceptance) [34]. In fact, adding BMT to the TLI/ATG protocol reduced, rather than increased, the percentage of tolerant animals.

A different approach has been taken by Storb et al. [40, 41] who developed a dog leukocyte antigen (DLA)-matched model of non-myeloablative BMT using cyclophosphamide and low-dose TBI for induction of mixed hematopoietic chimerism followed by a short course of immunosuppression to prevent GvHD and graft rejection. They subsequently reported on five mixed chimeric dogs who accepted kidney allografts from their DLA-identical hematopoietic cell donors long-term without immunosuppression, whereas kidney allografts transplanted in the opposite direction were promptly rejected [42]. In a recent study, they used the same protocol for DLA-identical BMT using two marrow donors per recipient, in the context of umbilical cord grafts. In this study, five of eight dogs were stable trichimeras, two were stable chimeras from one donor, and one rejected both grafts. Five of the seven chimeric dogs received kidney allografts from their hematopoietic cell transplantation (HCT) donors at least 6 months after BMT, and four of five grafts were accepted long-term without immunosuppression [43].

A miniature swine protocol achieved chimerism and renal allograft tolerance without TBI and thymic irradiation, using depleting antibodies and short-course cyclosporine A [44]. In another miniature swine model, stable mixed chimerism and long-term donor-type skin graft acceptance was achieved with 3 Gy TBI, 7 Gy thymic irradiation, T cell depletion with CD3-immunotoxin, and BMT followed by a 30-day course of cyclosporine A [17]. A similar protocol using ATG instead of CD3-immunotoxin was used for combined kidney and BMT in fully MHC-mismatched cynomolgus monkeys. Splenectomy was a necessary part of the protocol to prevent alloantibody production. With this protocol, long-term survival of fully mismatched kidney allografts was achieved in eight of 13 (62 %) monkeys overall and in eight of 11 (73 %) chimeric monkeys [45].

Of note, only animals that achieved mixed chimerism developed tolerance; however, most of them subsequently lost mixed chimerism without rejection of the kidney allograft. Attempts at reducing conditioning, for example by omission of splenectomy, failed in the regimen mentioned above since splenectomy seemed to be crucial in the establishment of B cell tolerance but a delayed kidney transplantation was possible [45]. Splenectomy was later successfully replaced with anti-CD154 monoclonal antibody but attempts to avoid thymic irradiation failed [46]. The same protocol used in the context of fully mismatched heart, instead of kidney, transplantation led to a prolongation of graft survival, but tolerance was not achieved [47].

In the above-mentioned nonhuman primate model, Kawai et al. [47] showed that each of the elements of this protocol was necessary to achieve tolerance to a fully mismatched kidney allograft. The thymic irradiation serves mainly to deplete donor-reactive mature T cells in the thymus, which are not depleted or tolerized by circulating anti-T cell-depleting antibodies, thereby permitting intrathymic

engraftment of tolerogenic donor-derived dendritic cells [10, 11, 48]. As thymic irradiation might be associated with delayed T cell recovery, especially in older individuals, efforts have been made to replace it with other modalities. In the mouse model, it has been demonstrated that the need for thymic irradiation can be overcome by the introduction of co-stimulatory blockade with one injection of either CTLA4-Ig or anti-CD154 mAb [20] or by a more intense course of T cell-depleting mAbs, which also inactivates alloreactive thymocytes [48]. However, when anti-CD154 was utilized in a mixed chimerism protocol in nonhuman primates, thromboembolic complications occurred [49]. Preliminary studies with costimulation blockade that targets the CD28 and the CD40 pathway, utilizes rapamycin, non-myeloablative doses of busulfan, and a short course of treatment with anti-IL-2R, have been reported to induce transient macrochimerism levels of more than 50 % [50].

12.3.7 Clinical Applications of Mixed Chimerism

The success in animal models of tolerance mechanisms involved with the achievement of durable-mixed chimerism via successful engraftment of allogeneic HSCs makes this a desirable approach for clinical application. In the clinical setting, proof-of-principle for successful tolerance induction by HSCT was provided by several reports of sequential allogeneic HSCT followed, much later, by a solid organ allograft from the same donor for a new indication [51–59]. Interestingly, some recent case reports showed that tolerance can also be achieved when an organ transplant is followed by BMT from the same donor [60–63].

However, this approach is not feasible and acceptable for the vast majority of organ allograft recipients because of the high complication rate, lethality, and cost of allogeneic chemo-radiotherapy based conditioning regimens, and the high risk of GvHD, even in the setting of HLA-identical BMT. Therefore, efforts have been undertaken to develop clinical protocols to establish mixed lymphohematopoietic chimerism with reduced intensity and therefore less toxic conditioning as well as methods to reduce the risk of GvHD.

The evaluation of reduced intensity conditioning protocols in patients with hematological malignancies undergoing allogeneic HSCT may provide the safety and toxicity data in humans needed for application of such protocols for solid organ tolerance induction with allogeneic HSC allografts. A large number of protocols using reduced intensity conditioning for HSCT in the setting of malignant disease have been published [64–68] (Table 12.2). While the goal was to develop less toxic treatments for patients with hematological malignancies, the results potentially apply to HSCT for induction of solid organ transplantation tolerance.

Currently, we are aware of three centers who systematically applied a reduced intensity conditioning regimen followed by HSCT for tolerance induction to living-donor kidney allografts as well as one center using a similar approach for

Table 12.2 Induction of mixed chimerism with reduced intensity HSCT protocols for treatment of hematologic malignancies

Center/# patients	Conditioning/stem cells	GvHD prophylaxis	Follow-up	Engraftment	GvHD
Stanford/37 [64]	TLI ATG HLA-identical PB	CSA MMF	Mean 482 days (222–1,069)	100 % initial MC Follow-up: 6/37 graft loss, 2 stable MC, 29 full donor chimeras	Acute $\geq$ Gr II: 1/37 Chronic 7/37
FHCRC/120 [65]	TBI Fludarabine HLA-identical PB or BM	CSA MMF	Mean 199 days	100 % initial MC Follow-up: 12/120 graft loss, In most increasing MC up to 180 days post-transplant	Acute $\geq$ Gr II: 67/120 Chronic: 42/120
MGH/42 [66]	CP, ATG TI (7 Gy) BM cells, HLA-identical	CSA DLI for patients with mixed chimerism and no GvHD	>300 days	100 % initial MC Follow-up: • *16 patients with MC received DLI* 10/16 full donor, 2/16 mixed chimeras, 4 graft losses • *26 patients without DLI* 5/26 full donor, 7/26 mixed chimeras, 10 graft losses, 4 NA	Acute $\geq$ Gr II: 12/26 without DLI, 7/16 with DLI Chronic: NA
MDACC/47 [67]	Fludarabine, CP, Rituximab, HLA-matched related (n = 45) or unrelated (n = 2)	Tacrolimus methotrexate	Mean = 60 months		Grade II–IV acute GvHD (11 %); Chronic GvHD (60 %);
MGH/12 [68]	CP, Siplizumab TI (7 Gy) BM cells (cohort 1 and 2), PB (cohort 3), HLA-mismatched haploidentical	CSA DLI for patients with MC and no GvHD	Up to 800 days	100 % initial MC Follow-up: • Cohort 1: 4 graft losses • Cohort 2: 2 full donor chimeras, 2 graft losses • Cohort 3: 1 full donor and 1 mixed chimera, 2 graft losses	Cohort 1: no GvHD Cohort 2: 2 acute $\geq$ Gr II, 1 chronic Cohort 3: 1 acute ‡ Gr II post-DLI

The table was adapted with permission from: [150]

ATG anti-thymocyte globulin, *BM* bone marrow, *CP* cyclophosphamide, *CSA* cyclosporine, *DLI* donor leukocyte infusion, *GvHD* graft versus host disease, *Gr* grade, *HLA* human leukocyte antigen, *MC* mixed chimerism, *MMF* mycophenylate mofitel, *PB* peripheral blood, *TI* thymic irradiation

living-donor liver allografts. Thus far, only one published trial conducted at the Massachusetts General Hospital in Boston has achieved long-term acceptance of human leukocyte antigen-mismatched kidney allografts without chronic immunosuppressive therapy [7, 69–71]. The results are summarized in Table 12.3.

12.4 Split Tolerance

It is clear that even with hematopoietic chimerism, it is possible for donor organs to be rejected (a phenomena known as "split tolerance"). Importantly, split tolerance appears to be more likely with mixed hematopoietic chimeras [72–74] than full chimeras. Most past studies demonstrated split tolerance in chimeras that maintained donor hematopoietic cells but rejected donor skin transplants, the cause of which was likely immunity toward polymorphic tissue-specific antigens expressed by donor skin but absent from their bone marrow cells [72, 74–80]. Although split tolerance involving tissues other than skin has not often been reported in murine chimeras, in a canine model, split tolerance was observed in which chimeric recipients rejected donor hearts [81]. Furthermore, pancreatic isoantigens were identified in rabbits. These findings suggest the possibility that split tolerance involving non-skin antigens may occur. In addition, potential host resistance to tolerance induction, or a general defect in self-tolerance, could lead to a state of split tolerance with or without involvement of donor tissue-specific antigens.

Many current studies are specifically designed to avoid the issue of split tolerance by using donor and recipient combinations matched for mH antigens [4, 5, 82–84]. The prevention of split tolerance by mH antigen matching could result from a number of mechanisms, including reduced indirect reactivity to the donor or elimination of allelic tissue-specific antigens. Although split tolerance can be eliminated or reduced by mH antigen matching [72], this artificial approach cannot be applied practically to clinical transplantation. Therefore, it is necessary to overcome the obstacle of 'split tolerance' before further strategies utilizing non-myeloablative conditioned mixed chimerism can be translated to the clinic.

12.5 Human ES Cells

12.5.1 Origin and Pluripotent Nature of hES Cell Lines

Human ES (hES) cells, similar to nonhuman primate and mouse ES cells, are derived from the inner cell mass (ICM) of the human blastocyst [85] by methods such as immunosurgical or laser isolation of the ICM and ex vivo culture [86]. ICM cells give rise to hES cell lines that proliferate in vitro, and maintain

Table 12.3 Induction of mixed chimerism with reduced intensity HSCT protocols for achievement of organ allograft tolerance in patients

Center/# patients/ organ transplanted	Protocol	Immune suppression and GvHD prophylaxis	Follow-up	Engraftment	GvHD	Organ outcome
Stanford/4/kidney [142, 143]	ATG, TLI PBSC $CD34^+$, HLA-mismatched	CSA, pred	Up to 3 years	3/4 with macro-chimerism	None	2/4 weaned of all immunosuppression, with subsequent Banff I rejection
Stanford/3/kidney [6]	ATG, TLI PBSC $CD34^+$ HLA-identical	CSA, MMF, Pred	Up to 2 years	2/3 with macro-chimerism (1 transient MC)	None	1/3 weaned of immunosuppression and tolerant 1/3 weaned with subsequent rejection
MGH/6/kidney [69–71]	CP, ATG TI (7 Gy) BM HLA-identical	CSA DLI for patients with MC and no GVHD	Up to 9 years	6/6 with initial macro-chimerism	2/6 GvHD 100 % donor chimera, 1 after DLI	6/6 tolerant (2/6 as full donor chimeras) 1/6 cellular rejection resolved
MGH/5 kidney [7]	CP, Rituximab, Siplizumab Pred TI (7 Gy), BM HLA-mismatched	CSA	Up to 5 years	5/5 with initial macro-chimerism All lost by 3 weeks	0	4/5 tolerant, one graft loss due to antibody-mediated rejection
India/33/kidney [144, 145]	CP, Intrathymic transplantation of donor renal tissue BMC and PBSCs 5 HLA-matched, 28 HLA-mismatched	CSA, pred	Up to 210 days	N/A	N/A	4 patients weaned from all immunosuppression and rejection-free by day 210
Belgium/2/liver [146, 147]	CP, ATG CD34 + PBSC HLA-mismatched Liver	tacrolimus or rapamycin, pred	370 and 270 days	1 Transient MC, 1 no MC	None	No rejections

The table was adapted with permission from: Fehr T, Sykes M. Clinical experience with mixed chimerism to induce transplantation tolerance. Transpl Int. 2008 Dec;21(12):1118–1135
ATG anti-thymocyte globulin, *BM* bone marrow, *CP* cyclophosphamide, *CSA* cyclosporine, *DLI* donor leukocyte infusion, *GvHD* graft versus host disease, *Gr* grade, *HLA* human leukocyte antigen, *MC* mixed chimerism, *MMF* mycophenylate mofitel, *PB* peripheral blood, *TI* thymic irradiation

pluripotency and high telomerase activity. Each hES cell line is normally derived from a single blastocyst, and is therefore genetically unique from the other hES cell lines available.

hES cells have the capacity to develop into the three embryonic germ layers of definitive ectoderm, mesoderm, and endoderm that give rise to all types of somatic cells. Upon injection into immunodeficient mice, hES cells generate teratomas composed of multiple tissue types [87], and express the pluripotent genes NANOG, SOX2, and OCT4, indicative of their pluripotent potential. In vitro, hES cells are capable of differentiating into multiple tissue types, including HSCs and mature hematopoietic cells, comprising erythroid, myeloid, and lymphoid lineages [88–107], and therefore may provide alternative sources of human hematopoietic cells for transplantation.

12.5.2 Current Progress in Derivation of Hematopoietic Cells from hES Cell Lines

Various methods for the differentiation of ES cells into specific hematopoietic lineages have been developed using mouse ES cells (mES cells). These methods have been adapted for use with hES cells, which have the possibility of being employed in regenerative medicine, and include growth and expansion as pluripotent undifferentiated cells on a supportive stromal cell layer [88–90, 101, 108–111] or on a basement membrane matrix, such as matrigel [112, 113]. Maintenance of hES cell cultures currently involves hand-picking of pluripotent colonies during passage to prevent overgrowth of differentiated cells. Once a renewable hES cell line is established either using a feeder layer or on a matrix with embryoid body (EB) formation, differentiation toward HSCs or hematopoietic-derived cells involves alteration of media such as addition of cytokines or growth factors or coculture with other mature marrow-derived cells [93, 98, 114–120].

Despite the different procedures applied in studying hematopoietic development from hES cells, different groups have achieved considerable common outcomes. First, different groups independently found that hematopoietic development from hES cells displays a specific pattern of timing after EB development or coculture, and that hematopoietic cells develop in clusters, as opposed to single independent cellular differentiation events occurring randomly within EBs or on stromal coculture [88, 93, 101, 114, 116, 117]. Second, during early hES cell differentiation, hematopoietic cells are derived from CD45-precursors that co-express CD31 and CD34 surface markers [88, 93, 101, 114, 117]. Two groups have identified an immature endothelial population as being responsible for giving rise to hematopoiesis from hES cells [93, 117].

Although the validity of ES cells as a model for adult hematopoiesis is controversial because long-term engraftment of HSCs has not been demonstrated from hES cells, preliminary data suggest that hES cell-derived hematopoietic cells have

HSC properties when injected into immunodeficient murine recipients [108, 111, 118]. Still, the ability to generate fully functional hES cell-derived HSCs capable of long-term multilineage reconstitution through serial transplantation has not yet been demonstrated. Before applying to clinical patient care, it is generally accepted that in order to avoid transmission of potential xenogenic pathogens, hES cells should be maintained in culture and differentiated without exposure to animal product(s). Completely animal free conditions for all steps involved in manipulation of hES cells, that is isolation, cryopreservation, propagation of pluripotent cells in culture, and ex vivo differentiation to a defined lineage, have not yet been published.

12.5.3 Approaches to Hematopoietic Differentiation from hES Cells

In the first studies, $CD34^+$ hematopoietic precursor cells expressing hematopoietic transcription factors were derived from hES cells using a coculture method with the murine BM stromal cell line S17 or the yolk sac endothelial cell line C166 in the presence of fetal bovine serum (FBS), without any other added cytokines or growth factors (Table 12.4) [88]. $CD34^+$ cell selection leads to enrichment of the hematopoietic progenitor cells capable of forming characteristic myeloid, erythroid, and megakaryocytic colonies from clonogenic progenitors. Hematopoietic cells derived from these colonies also expressed typical hematopoietic cell morphology and phenotype.

Multiple reports have subsequently used a variety of stromal cell lines, including OP9, M2-10B4, FH-B-Htert, and primary human BM stroma, to support hematopoietic development from hES cells [89, 90, 101], however, the overall extent of hematopoietic development is low (0.1–2 % of $CD45^+$ during 8–20 days of differentiation). This is also reflected in vivo, where hES cell-derived progenitors from coculture with S17 stromal cells engraft primary and secondary fetal sheep recipients with very low levels of chimerism (0.001–0.05 % of the bone marrow) [111].

More recent studies have evaluated what soluble or secreted "factors" are important for hematopoietic development in these cultures. Ledran et al. [91] demonstrated that coculture of hES cells on AM20.1B4 cell line lead to increased development of severe combined immunodeficient (SCID) mouse-repopulating cells (SRCs), a close surrogate for HSCs. Further analysis showed AM20 cells produced more TGFβ than other stromal cell lines tested, and the addition of exogenous TGFβ to hES cells cocultured with other stromal cell lines had a similar effect. The ability to modify stromal cells also provides a useful means to define specific "niche factors" that regulate human hematopoiesis.

For example, hES cells express Frizzled receptors for Wnt protein, and expression of Wnt1 on S17 and M2-10B4 cells leads to increased hemato-endothelial cell

Table 12.4 Differentiation of human ES cells into hematopoietic cells

Ref	hESC-derived hematopoietic cell	hESC culture conditions/comment
[88]	CD34+ HSC	cocultured with S17 or C166 and FBS
[89]	CD34+ HSC	coculture on FH-B-hTERT
[90]	CD34+CD45 + HSC	coculture with S17, SCF, TPO, and Flt3L
[91]	CD34+ HSC	coculture with AGM and TGF-beta1 and TGF-beta3
[92]	CD34(bright)CD31(+)Flk1(+) (endothelial and hematopoietic stem cell)	coculture with stromal cells that express Wnt1
[93]	PFV+ cells (primitive endothelial-hematopoietic progenitors)	EB formation by culture on matrigel with cytokines and BMP-4
[94]	KDR+ hemangioblasts (form hematopoietic and endolethial cell)	BMP-4 stimulated EB
[95]	CD43+ (leukosialin) HPC	coculture with OP9 stromal cells
[96]	Monocytes and macrophages	EB formation with M-CSF and IL-3
[97]	$ACE^{+}CD45^{-}CD34^{+/-}$ hemangioblasts	EB formation with BMP-4 and cytokines
[98]	CFC-E, CFC-M	EB formation with growth factors
[99]	Erythroid	coculture on murine FLSC
[100]	Erythroid	EB formation with BMP-4, VEGF, bFGF
[101]	CD34+ HSC B cells	coculture OP9 cells. culture hESc-derived CD34+ cells on MS-5 stromal cells with SCF, Flt3L, IL-7, and IL-3
[102]	T cell	coculture with OP9-DL1 with FLT3-L, IL-7 SCF
[103]	T cell	coculture with OP9 cells and implant in SCID-hu mouse
[104]	T cell	EB formation with BMP4, SCF, Flt3L, and implant in SCID-hu mouse
[105]	NK cell	hESC-derived cells lymphoid differentiation favors the NK-cell lineage not T or B cells.
[106]	Megakaryocyte, Platelet	coculture OP9 cell with TPO
[107]	Megakaryocyte, Platelet	coculture OP9 cells with VEGF and TPO

ACE angiotension converting enzyme, *AGM* murine stromal cell lines from the aorta-gonado-mesonephros region, *bFGF* basic fibroblast growth factor, *BMP-4* bone morphogenetic protein-4, *C166* yolk sac endothelial cell line, *CFC-E* colony-forming cells-eryhtroid, *CFC-M* colony-forming cells-macrophage, *EB* embryoid body, *ESC* embryonic stem cells, *FBS* fetal bovine serum, *FH-B-hTERT* a human fetal liver-derived cell line, *FLSC* fetal liver stromal cells, *Flt3L* fms-like tyrosine kinase 3 receptor ligand, *HPC* hematopoietic progenitor cell, *HSC* hematopoietic stem cell, *IL-3* interleukin-3, *IL-7* interleukin-7, *KDR* kinase insert domain protein receptor, *M-CSF* macrophage colony stimulating factor, *OP9* a bone marrow stromal cell line, *OP9-DL1* OP9 cells expressing high levels of Delta-like 1, *PFV*+ positive for PECAM-1, FlK-1, and VE-cadherin, *S17* murine bone marrow stromal cell line, *SCF* stem cell factor, *SCID-hu* a chimeric mouse that contains small pieces of human fetal liver and thymus under the renal capsule of severe combined immunodeficient (*SCID*) mice, *TPO* thrombopoietin, *VEGF* vascular endothelial growth factor

development from hES cells [92]. Another recent report by Bhatia and colleagues [121] has shown noncanonical Wnt signaling also plays a key role in this system. EB-induced differentiation of hES cells can result in hematopoietic colony-forming

cells (CFCs), and this can be further enhanced by BMP4 (bone morphogenetic protein-4) and a cocktail of cytokines to expand bone marrow progenitor cells [94, 114]. Furthermore, it has been shown that hematopoiesis from hES cells may arise from primitive endothelial-like cells that express PECAM-1, FLK-1 (KDR), and VE-Cadherin, suggesting the existence of an embryonic endothelium that possesses hemagioblastic characteristics [93]. Numerous other studies have also used EB-mediated differentiation for development of both hematopoietic progenitor cells and more mature cell populations from hES cells [96, 97]. Notably, studies by several investigators, including Elefanty's group and Zandstra's group, who use EB-mediated differentiation of hES cells, highlight methods to make EB-mediated hematopoietic differentiation of hES cells more consistent and efficient [98, 119, 122].

12.5.4 Development of Hemato-Endothelial Cells from hES Cells

hES cells provide an unparalleled means to analyze early stages of human development that are otherwise difficult to study in a systematic manner. Several groups have characterized progenitor cells with both hematopoietic and endothelial cell potential (termed hemogenic-endothelium or hemangioblasts) from hES cells [92–95, 117]. Although this characterization of hemato-endothelial cells from hES cells has used different hES cell lines and different methods to induce or support differentiation, the timing of development and phenotype of this cell population is reasonably similar. Wang and colleagues [93] first characterized a population of $CD31^{+}Flk1(KDR, CD309)^{+}VE\text{-}Cadherin(CD144)^{+}CD45^{-}$ cells with the use of EB-mediated differentiation. Similarly, Woll et al. [92] used stromal cell-mediated differentiation to generate CD34-bright $CD31^{+}Flk1(KDR)^{+}CD45^{-}$ cells with hemato-endothelial potential.

Key studies by Zambidis et al. [97] identified angiotensin converting enzyme/CD143 (as recognized by the BB9 antibody) on hES cell-derived hemangioblasts as not just a lineage "marker," but also as a functionally important molecule to regulate hemato-endothelial cell development by the renin-angiotensin system. Inhibiting angiotensin-converting enzyme activity (by captopril) or blocking Angiotensin Two Receptor type 1 (AGTR1) led to decreased hematopoietic development and increased endothelial cell development from hEB-derived progenitors. In contrast, blocking Angiotension Two Receptor type 2 (AGTR2) leads to a 5-fold increase in hematopoietic progenitor cells (CFCs) and increased hematopoietic development from hES cell-derived clonal hemangioblast colonies (BL-CFCs). Therefore, these analyses not only provide insight into regulation of early human hematopoiesis, but they may also be applicable to clinical hematopoietic cell transplantation when prompt hematopoietic engraftment is desired and may be affected by patient medications. Keller and colleagues [123], who pioneered studies to characterize development of hemangioblasts from mES cells, have also isolated 2 separate populations of cells from hES cells that meet the criteria of hemangioblasts (i.e, blast colonies having both hematopoietic and vascular potential) [94].

The OP9 stromal cell line (genetically deficient in M-CSF production) commonly used for studies of hematopoiesis from mES cells has also been used for hES cell-based studies. Vodyanik et al. [95] demonstrated that early progenitors committed to hematopoietic development could be identified by surface expression of leukosialin (CD43). The appearance of CD43 was found to precede that of CD45 on all types of emerging clonogenic progenitors, and CD43 can reliably separate the hematopoietic $CD34^+$ population from $CD34^+$/CD43-endothelial and mesenchymal cells. Interestingly, a population of $CD34^+$/CD43-KDR^+ cells with dual hemato-endothelial potential was isolated, similar to the CD34 bright $CD31^+Flk1(KDR)^+$ cell population isolated by Woll et al. [92]. With the use of stromal cell lines engineered to express mediators of canonical and non-canonical Wnt signaling, this group further tested the role of Wnt proteins to provide insight into mechanisms that mediate hES cell-derived hemato-endothelial cell development. Wnts are known to play a key role in many developmental pathways [124]. Stromal cell expression of Wnt1 (activating canonical Wnt signaling pathway), but not Wnt5 (non-canonical Wnt signaling) lead to increased CD34 bright $CD31^+Flk1^+$ hemato-endothelial cells and CD34-dim $CD45^+$ hematopoietic progenitor cells [92]. A corresponding decrease in these cell populations was shown by the inhibition of canonical Wnt signaling.

12.5.5 ES Cell-Derived Hematopoietic Cells Evaluated for Long-Term Engraftment Potential

Isolation of putative HSCs from hES cells with long-term multilineage in vivo engraftment potential could be considered a straightforward goal. However, studies to date continue to remain challenging especially with hES cells. In contrast, there are reports of murine ES cell-derived hematopoietic elements capable of long-term multilineage in vivo engraftment [125–128].

12.5.5.1 Murine ES Cell-Derived Hematopoiesis in Animal Models

Our group reported the formation of $ckit^+$/$CD45^+$ transplantable hematopoietic progenitors, capable of in vivo multilineage reconstitution of irradiated mice, following culture of mES cells in methylcellulose in the presence of serum, stem cell factor, IL-3, and IL-6 [129]. Despite transplantation into allogeneic recipients, these mES cell-derived HSCs, engrafted and generated extensive hematopoietic chimerism and contributed to both the myeloid and lymphoid lineages. Importantly, direct delivery to the bone marrow via intrafemoral instillation enabled significantly higher numbers of engrafted cells, as compared to intravenous application in the tail vein [129]. These findings suggest that providing direct contact with the niche may be especially important for developmentally immature

stem cells. Interestingly, applying renewable high numbers of purified c-kit$^+$/CD45$^+$ cells enabled engraftment even in MHC-mismatched mice, without signs of graft rejection or induction of GvHD. Tolerance induction through high numbers of transplanted cells may be another advantage of ES cell-derived material. However, the encouraging results reported in our study have not been yet independently replicated.

In a more recent study, we evaluated whether mES cell-derived HSC could produce islet cell tolerance, a phenomenon termed graft versus autoimmunity (GVA), without causing GvHD. We demonstrated that ES cell-derived HSC may be used to prevent autoimmune diabetes mellitus in NOD mice without GvHD or other adverse side effects. ES cells were cultured in vitro to induce differentiation toward HSC, selected for c-kit expression, and injected either i.v. or intrabone marrow (IBM) into sublethally irradiated NOD/LtJ mice. Nine of 10 mice from the IBM group and 5 of 8 from the i.v. group did not become hyperglycemic, in contrast to the control group, in which 8 of 9 mice developed end-stage diabetes. All mice with >5 % donor chimerism remained free of diabetes and insulitis, which was confirmed by histology. Splenocytes from transplanted mice were unresponsive to glutamic acid decarboxylase isoform 65, a diabetes-specific autoantigen, but responded normally to third-party antigens. We concluded that mES cell-derived HSC can induce an islet cell tolerizing GVA effect without GvHD. This study represents the first instance of ES cell-derived HSC cells treating disease in an animal model.

Several groups have exploited different strategies such as overexpression of homeobox B4 gene (HoxB4) that promotes HSC development or other genes in mES cell-derived cells to enable development of cells capable of long-term, multilineage hematopoietic cell engraftment [36–38]. Similar strategies have been less successful for improving development of hematopoietic cells capable of long-term multilineage engraftment from hES cells [118, 120, 130]. Another problem that has not been clearly resolved is whether HoxB4-expressing cells might induce tumors due to the retroviral vectors. Although no-one has reported tumors in recipients of these cells, Zhang et al. [131] reported that there was a high incidence of leukemia in dogs that received HoxB4-expressing cells. These results indicate the need for alternative approaches to genetic modification with HoxB4.

12.5.5.2 Human ES Cell-Derived Hematopoiesis in Animal Models

Several studies have evaluated the engraftment potential of hES cell-derived hematopoietic cells (Table 12.5) [91, 108, 111, 118]. The first used an intra-BM transplantation (IBMT) technique to successfully engraft hES cell-derived cells in 11 of 19 mice [118], however, evidence of human reconstitution was limited compared with UCB-derived cells. Interestingly, this same study was unable to show successful engraftment after intravenous (tail vein) injection of the mice with hES cell-derived hematopoietic cells. Indeed, there was actually a decrease in

Table 12.5 Human ES cell-derived hematopoiesis in animal model

Author (reference)	hESC-derived cells	Recipient animal model	Method of ESC-derived cell deliver (IBM, IV)	% human hematopoiesis	Complications teratomas, GvHD, etc.
Gori et al. [148]	hESC-derived $CD31^+$, $CD34^+$, $CD45^+$ cells	NOD/SCIDγ_c^{null} female mice	Intravenous injection	<1 %	Teratomas
Park et al. [132]	hESC-derived $CD13^+$, $CD19^+$, $CD34^+$, $CD45^+$ cells	Chicken embryos	Yolk sac Injection	0.04–2 %	None
Tian et al. [135]	hESC-derived $CD34^+$ cells	NOD/SCID$\gamma_c^{-/-}$ mice	Directly inject to liver	0.03–15.2 %	Mesenchymal tissue
Ji et al. [126]	hESC-derived $CD34^+$, $CD45^+$ cells	NOD/SCID & NOD/SCID$\beta^{-/-}$ mice	Femur and intrahepatic injection	Not detectable	Unknown
Narayan et al. [111]	hESC-derived $CD34^+$, $CD38^+$ cells	Fetal sheep	Peritoneal cavity injection	0.1–0.2 %	Unknown
Tian et al. [108]	hESC-derived $CD34^+$, $CD45^+$ cells	NOD/SCID mice	Intravenous injection	<1 %	None
Wang et al. [149]	hESC-derived $CD31^+$, $CD34^+$, $CD45^+$ cells	Rag2$^{-/-}\gamma_c^{null}$ female mice	Intravenous injection	<1 %	Unknown
Wang et al. [118]	hESC-derived $CD34^+/CD45^+/CD38^-$ cells	NOD/SCID & NOD/SCIDβ2 m$^{-/-}$ mice	Femur and intravenous injection	1 %	Unknown
Ledran et al. [91]	hESC-derived $CD45^+$ cells	NOD/LtSz-ScidIL2R γ^{null}	Femur and intravenous injection	0.1–16 %	Unknown
Swijnenburg et al [137]	hESC-derived $CD3^+$ T cells	NOD/SCID mice	Gastrocnemius muscle injection	2–5 %	None

survival of the mice after intravenous injection, probably because of aggregation of the cells after injection, resulting in pulmonary emboli.

Tian and colleagues [108] used both IBMT and intravenous injection of hES cell-derived hematopoietic cells to demonstrate successful engraftment in the mice without any decreased survival or pulmonary emboli after intravenous injection. These results probably reflect differences in the cell populations derived by alternative methods (by coculture with stromal cells versus by EB formation). In this study, BM analyzed 3 or more months after intravenous injection showed an average of 0.69 % human $CD45^+$ cells, still considerably reduced compared with mice injected with cells derived from UCB. In mice in which IBMT was used, the level of engraftment was seen to be approximately 2 % in the femur directly injected with the cells and essentially the same in the contralateral femur. The engrafted cells were primarily $CD45^+/CD33^+$ myeloid cells; however, some $CD34^+$ cells were also seen, suggesting HSC survival. Secondary transplantation studies were done to more rigorously show successful engraftment, although at a level only detectable by polymerase chain reaction (PCR) analysis in the secondary recipients.

NOD/SCID mice are reported as NK-cell deficient; however, several analyses have shown that these mice retain some NK cell activity [118]. In this regard, mice treated with anti-asialo GM1 antiserum (which depletes NK cells) led to a modestly enhanced level of engraftment, probably related to lower HLA class I molecule expression on the hES cell-derived progenitors compared with UCB-derived HSCs, a difference that would predispose them to NK cell-mediated lysis. In light of the residual immunity in NOD/SCID mice, a more recent study used the more immunodeficient NOD/SCID/IL2R$\gamma c^{-/-}$ mice. Here, Ledran et al. [91] also used a stromal cell coculture system and transplanted a heterogenous population of unsorted hES cell-derived cells into adult recipients. Coculture of hES cells with one stromal cell line (AM20.1B4) isolated from the aorta/mesenchyme region of day 10 mouse embryos led to higher levels of engraftment compared to the other stromal cell lines isolated from other developmental niches. Up to 16 % of human $CD45^+$ cell engraftment could be detected in the peripheral blood (PB) of some mice that received a transplant, although engraftment in the BM remained at only 1–2 %.

There were at least two other important findings in these studies that compared efficiency of different mouse stromal cell lines on hematopoietic development from hES cells [91]. First, the stromal cell lines that lead to the highest level of hematopoietic development in vitro did not necessarily lead to the best SRC development. For example, hES cells cocultured with the cell line UG26.1B6 produced more $CD34^+$ cells and more CFCs in vitro, compared with hES cells cocultured with AM20.1B4 cells. However, the AM20-derived cells were markedly better at in vivo engraftment. Second, stromal cell expression of TGFβ superfamily members correlated with improved hematopoietic ($CD45^+$ cell) development from hES cells. Addition of TGFβ1 and TGFβ3 led to improved $CD45^+$ cell development, although studies to evaluate the SRC potential of the TGFβ-treated cells were not reported [91].

Narayan et al. [111] used a fetal sheep transplantation system; hES cell-derived hematopoietic cells were injected in utero into the peritoneal cavity of fetal sheep at less than 65 days of gestation. 5–17 months after birth, approximately 0.1 % human $CD34^+$ or $CD45^+$ cells were seen in the BM and/or peripheral blood (PB). Again, this level of engraftment is decreased compared with the use of UCB-derived cells [111]. Low levels of engraftment was confirmed by PCR for human DNA from BM samples in 6 of 8 animals analyzed at 33–39 months after transplantation. Furthermore, human hematopoiesis in sheep that received a secondary transplant was followed for up to 22 months.

In another animal model, hES cell-derived $CD34^+$ cells were transplanted into chick embryos [132]. Here, human $CD45^+$ cells could be seen to develop, with highest levels in the bursa of Fabricius, including $CD19^+/IgM^+$ cells consistent with B cells. Other erythroid, myeloid, and endothelial cell populations could also be identified by phenotype, although no T cells were found. Although these studies are limited to a short time course, this does provide an intriguing in vivo model for future analyses to better identify mediators of hematopoietic development and engraftment.

To produce HSCs with better long-term multilineage engraftment potential from hES cells, it is probably important to develop culture techniques that more closely resemble the in vivo microenvironment necessary to stimulate a genetic program needed for not only the hematopoietic specification of the hES cells, but also for the transition from primitive to definitive hematopoiesis. In this regard we need better understanding of the pathways involved in this complex process. Several signaling pathways, including Wnt, Notch, Hedgehog, and TGFβ/Smad, are likely to play a prominent role in this developmental process [115, 133, 134]. Furthermore, the in vivo environment may skew development of the hES cell-derived cells, as shown by recent studies that used luciferase (luc)–expressing hES cells. Here, transplantation of luc^+/hESC-derived $CD34^+$ cells, that are known to have both hematopoietic and endothelial potential in vitro, lead to long-term engraftment of the luc^+ cells when transplanted into neonatal NOD/SCID/IL2R$\gamma c^{-/-}$ recipient mice, as visualized by bioluminescent imaging. Although the engrafted luc^+ ES cell-derived cells could be seen to expand and migrate systemically to diverse anatomic regions for several months after transplantation, analysis of the surviving, and expanding cells found them to be mainly endothelial cells with little long-term hematopoietic cell engraftment [135]. In another report, Lu et al. [136] also transplanted hES cell-derived cells with hemato-endothelial cell potential into xenogeneic models of vascular injury and demonstrated endothelial cell engraftment and repair, without evidence of hematopoietic engraftment.

One important and often overlooked outcome of these multiple studies concerns the safety of hES cell-based therapies because of the ability of the undifferentiated hES cells to form teratomas on injection into animals. To date, no teratoma formation has been seen in any engraftment studies that used hematopoietic progenitors derived from hES cells. This is despite the use of irradiated immunocompromised mice that are very susceptible to teratoma development.

Nevertheless, rigorous assays for residual pluripotency and or teratoma formation would be needed to prove that transplantation of hES cell-derived hematopoietic cells pose no risk of teratoma development before clinical application could proceed.

Because most hES cell-derived cells have been tested in preclinical models using immunodeficient mice and rats, the actual immune response against the hES cell-derived cells is difficult to determine. Cells derived from hES cells would almost certainly be an allogeneic tissue source and be subject to typical immune rejection [137]. Recent studies have shown that hES cells and hES cell-derived cells can be rejected by an adaptive T cell mediated process [137], and other studies have shown that innate immune effectors (NK cells) can also mediate rejection of hES cell-derived hematopoietic cells that may have low levels of HLA class I expression [108]. Multiple methods have been proposed to inhibit the host immune response against these transplanted tissues [138, 139]. These options range from standard pharmaceutical immunosuppressive drugs to more creative means to prevent immunorejection such as decreasing HLA expression or promoting expression of immunosuppressive molecules.

12.6 Conclusion

The therapeutic potential of allogeneic HCT is vast, with potential to induce immune tolerance to allografts and xenografts. The development of reduced intensity regimens for achieving allogeneic hematopoietic engraftment across major histocompatibility barriers and its recent application in clinical trials of kidney transplantation is encouraging. A deeper understanding of the mechanisms by which HSCT overcomes pre-existing allo- and autoimmunity will permit the development of even more successful and less potentially toxic approaches to exploiting this capacity.

ES cells provide new opportunities for developing and establishing novel treatments, including induction of transplantation tolerance, because of their unique characteristics: lack of MHC antigens, poor expression of costimulatory molecules, lack of T cells that can trigger GvHD, availability to repeatedly infuse large number of ES cell-derived HSC, and to generate composite tissue allografts of ES cell-derived HSC, dendritic cells, Treg cells, MSCs, etc. For these reasons, conditioning recipients of allogeneic ES cell-derived HSCs could potentially be safer and less rigorous than conditioning recipients of bone marrow cells. However, in vitro, differentiation of ES cells into specialized cells and tissues including hematopoietic cells has remained a challenge due to still evolving optimal methods of hematopoietic cell differentiation, requirement of ES cell lines and ES cell-derived HSC to be free of exposure to animal products, and development of sensitive assays to screen for potential of teratoma development from any ES cell-derived product.

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Chapter 13
Prospects for the Induction of Transplant Tolerance Using Dendritic Cells

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Abstract Dendritic cells (DCs) play a key role in both central and peripheral tolerance induction and maintenance. Strategies to modify dendritic cells ex vivo to induce tolerance to an allograft have been extensively studied. Approaches include genetic modification of DCs, siRNA silencing of co-stimulatory pathways and drug modification. The ex vivo approaches are associated with the generation of DCs that can induce hypo-responsiveness in responder T cells and/or the expansion or de novo generation of regulatory T cells. However, in stringent models of transplantation they fail to reliably induce long-term allograft survival. We explore the mechanisms underlying this lack of efficacy and other potential strategies of DC modification including targeting of alloantigens to defined DC subsets such that we have reliable protocols to induce peripheral tolerance.

13.1 Introduction

Tolerance can be defined as the acceptance of antigenic donor tissue by a host with or without the need for continuous immunosuppression. The ultimate goal, however, is to achieve long-standing clinical allograft acceptance without the need for long-term immunosuppression. Current immunosuppressive agents such as calcineurin inhibitors act by blocking T cell activation, hence targeting the primary

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mechanisms of graft rejection and graft versus host disease (GvHD). Unfortunately, these drugs are associated with significant side effects, including hyperlipidemia, nephrotoxicity, diabetes and increased risk of opportunistic infections and malignancies [1]. Newer immunosuppressive drugs, used in the clinic, include humanised anti-IL-2 receptor mAb (daclizumab); mycophenolate mofetil (MMF), which blocks lymphocyte purine biosynthesis; and sirolimus (rapamycin), which inhibits multiple cell cycle regulators [2]. All of these agents have their own toxicities and so drug free or reduced drug therapies are the preferred future option. However, agents such as rapamycin, which do not block IL-2-dependent proliferation in the same way as calcineurin inhibitors, are thought to be permissive rather than inhibitory in the induction of tolerance, therefore, their use may be preferred in combination with a tolerance induction protocol [3, 4]. A word of caution though comes from a study in which rapamycin treatment reversed an established tolerant state induced following anti-CD3 treatment [5].

Altogether, the best strategy to induce tolerance is to manipulate the immune system itself, such that the use of immunosuppressive drugs can be eliminated completely. One cell type that has received a lot of attention for this purpose is the dendritic cell (DC) due to its pivotal role in immune responses.

13.2 Dendritic Cells: Subtypes and Functions

DCs are specialised antigen presenting cells (APCs) that have a crucial role in the regulation of immune responses; they are a heterogeneous group of cells differing in anatomical localisation and physiological function. There is a constant production of DCs throughout life from hematopoietic progenitors. Whilst the precursor origin of many peripheral cells has been well established, the origin of DCs remained elusive. Originally considered to be of myeloid origin (because of their relatedness to monocytes and macrophages) other evidence suggests that DCs may originate from at least two distinct hematopoietic lineages (myeloid and lymphoid). Because of the functional heterogeneity of DCs in both mouse [6–8] and human [9] it has been difficult to establish the identity of their common precursors. In murine bone marrow (BM) both common lymphoid progenitors (CLP) and common myeloid progenitors (CMP) are known to exist [10, 11]. Both these multipotent lineage-restricted cells can generate DCs, which are distinguishable by the presence or absence of IL-7Rα.

As mentioned above, heterogeneity exists within murine DCs, however, they can be simply divided into conventional DCs (cDC) present in spleen and lymph nodes, which are also referred to as ‘resident’ DCs, and the tissue-derived DCs, also known as ‘migratory’ DCs. In the spleen, subtypes of DCs have been described based on their CD4 and CD8α expression and consist of $CD4^{+}CD8\alpha^{-}$ and $CD4^{-}CD8\alpha^{+}$ subsets. The latter can be either $CD205^{+}$ or $CD205^{-}$ [12, 13]. In the lymph node an additional subtype of $CD4^{-}CD8\alpha^{lo}CD205^{+}$ cells are found with varying levels of CD11b expression [14]. The splenic populations of DC are

developmentally distinct, and are semi-mature in their resting state, as assessed by their expression of co-stimulatory molecules and their capacity to activate allogeneic T cells [15]. These subsets of DC are differentially distributed in the splenic pulp and exhibit varying migration properties in response to stimuli such as LPS [16]. As well as exhibiting heterogeneity phenotypically they also exhibit differences in their function. $CD8\alpha^+$ DCs have been shown to induce $CD8\alpha^+$ T cell responses whilst inhibiting $CD4^+$ T cells responses [6]. Once activated, $CD8\alpha^+$ DC produce much higher levels of IL-12 than $CD8\alpha^-$, providing a further functional distinction [6, 16]. Another population of DCs exists in both the spleen and LN, the plasmacytoid DCs (pDC). These cells are distinct from the cDCs [17]. Tissue-derived/migratory DCs have been characterised in the skin, lung and gut. DCs within the skin consist of Langerhans cells (LCs), dermal DCs and $CD103^+$ cells, while in the lung, $CD103^+$ DCs are also found, as well as another $CD11b^+$ DCs subset. $CD103^+$ DCs are also present in the gut. The classification and function of these cells have been described elsewhere by Heath and Carbone [18].

In humans, immature DCs are found in BM and blood, with differentiated phenotypes seen in both lymphoid and non-lymphoid tissues. In general, human DCs are classified as being lymphoid or myeloid in origin (both from $CD34^+$ precursors), with lymphoid DC giving rise to pDC ($CD11c^-CD14^-$) and myeloid precursors forming interstitial or LCs ($CD11c^+CD14^{+/-}$) (reviewed in [19]). The best-studied human DCs are those derived from monocytes. $CD11c^+CD14^+$ monocytes can give rise in culture to immature DCs under the influence of IL-4 and GM-CSF or TNFα [20–22]. In addition, $CD11c^+$ blood DCs can differentiate into LCs in the presence of TGF-β [23]. It is possible to generate macrophages from both monocytes and blood $CD11c^+$ DCs using GM-CSF or M-CSF, demonstrating a degree of functional plasticity in this system [9, 24]. $CD11c^-$ DCs (pDCs) die rapidly after isolation and are critically dependent on IL-3 for survival and CD40 ligation for maturation.

13.3 Dendritic Cells and Tolerance Induction

There are several lines of evidence that suggest that DCs may be useful tools in achieving peripheral tolerance. One key feature of DCs is their excellent capacity for migration to T cell areas of lymphoid tissues. When in a mature state, it is here that DCs prime naïve T cells [25, 26]. Naïve T cells are known to contribute to alloresponses and graft rejection [27, 28]. The depletion of naïve T cells has been shown to prolong allograft survival or reduce graft versus host (GVH) disease [29, 30]. The capacity of DCs to migrate to the primary sites where alloresponses arise offers the opportunity to manipulate DCs to directly inhibit allospecific T cell activation. However, immature DCs can also reach the lymphoid tissues and, by interacting with T cells, can contribute to tolerance induction. In mice, both $CD8\alpha^-$ and $CD8\alpha^+$ DCs promote apoptosis of alloreactive T cells via expression of FasL [31, 32]. DC expression of other death-inducing ligands [e.g. tumour necrosis factor

(TNF)-like receptor apoptosis-inducing ligand (TRAIL)] might also allow direct deletion of alloreactive T cells. In addition, there is experimental evidence to suggest that DCs in the steady state (in the absence of deliberate exposure to maturation signals) can tolerate peripheral $CD4^+$ and $CD8^+$ T cells by inducing anergy or regulation, depending on the model system studied [33–35]. It is thought that these are important physiological processes designed to limit the pool of autoreactive T cells that might otherwise cause disease.

13.3.1 Tolerogenic DCs: Definition

Defining what constitutes a tolerogenic DC remains problematic. Different factors contribute to a tolerizing interaction with T cells, from the DCs capacity to present the relevant antigen and the level of co-stimulation and co-inhibition that occur, to the capacity of the responding T cell to translate these signals into a tolerant state.

13.3.1.1 Immature = Tolerogenic DCs?

There has been a tendency to apply the terms immature and tolerogenic DCs synonymously. Probst et al., using DC-specific inducible expression of T cell epitopes by recombination (DIETER) mice [36], demonstrated that steady-state DCs can induce $CD8^+$ T cell tolerance to cytomegalovirus (CMV) dominant epitopes in vivo but that in the presence of CD40 stimulation immunity reliably occurs, in keeping with the conventional paradigm that maturity equals immunity. However, matured DCs can also function as tolerogenic DCs [37]. Immature DCs can invoke anergy in responder T cells due to sub-optimal co-stimulation and limited antigen presentation capacity in vitro. Within secondary lymphoid organs, as described above, immature DCs process and present antigens without exposure to maturational stimuli making immature-type DCs ideal cells to induce anergy to self-antigens suggesting that an immature DC phenotype could be tolerogenic. Following maturation, however, DCs are more effective at presenting antigen on major histocompatibility complex (MHC) class I and II. If the T cell that encounters a DC has a low avidity for the antigen, low levels of antigen presentation may be insufficient to drive a response and in this setting a more mature DC might be required.

13.3.1.2 Co-stimulatory and Co-inhibitory Molecules

The nature of the immune response following the interaction between DCs and T cells with varying levels of antigen presentation may then crucially depend upon the balance of co-stimulation and co-inhibition and the intrinsic properties of the responding T cell. It has been known for a long time that lack or low levels of CD80 and CD86 expression (signal 2) lead to anergy [38]. However, DCs

expressing substantial levels of co-stimulatory molecules and other maturation markers can be tolerogenic [39, 40]. For example, in an antigen-specific mouse model, DCs from $CD40^{-/-}$ and $CD40L^{-/-}$ mice did not elicit $CD4^+$ or $CD8^+$ T cell immunity, even though the DCs presented antigen on MHC class I and II, and expressed high levels of CD80/86. In addition, the indirect activation of DCs by inflammatory mediators results in the usual phenotypic markers of a mature DC, and whilst able to support the clonal expansion of $CD4^+$ T cells, they were not capable of polarising the T cell response, i.e. they do not form IFNγ producing effector cells in an otherwise Th1 model [41]. Furthermore, the traditional paradigm of signal 1 and signal 2 may require additional thought in the context of tolerogenic DCs. Whilst the B7 family members CD80 and CD86 are traditionally thought of as archetypal providers of signal 2, they also provide a negative signal via CTLA-4. Other members of the CD28–B7 families, expressed by DCs including programmed cell death-ligand 1 (PD-L1) and ICOS-ligand, also play critical roles in immune regulation [36, 42, 43] Similarly, other cell surface molecules such as OX40 ligand, 4-1BB ligand and CD70 all contribute to immunity but can also have an inhibitory effect on T cell activation [44]. Finally the immunoglobulin–like transcript family (ILT), in particular ILT-3, has been shown to be important in the generation of inducible Tregs from naïve T cells, and this pathway may be more significant for resting DCs as opposed to drug modified DCs [45, 46]. In the quest for a tolerogenic DC, it appears that we require a cell type that has the appropriate quantitative differences in expression of cell surface stimulatory and inhibitory molecules as well as a specific cytokine production profile (IL-12/IL-10 balance in favour of IL-10).

13.3.1.3 Anergy/Regulatory T Cells

So far, we have described tolerogenic DCs as DCs capable of inducing anergy in the responder T cells. However, tolerance induced by DCs may also arise due to the expansion or de novo generation of regulatory T cell (Treg cell) subsets. While anergy is often the consequence of sub-optimal co-stimulation and/or increased co-inhibition, the expansion or induction of regulatory Treg cell subsets requires activation and adequate co-stimulation. Indeed, the levels of co-stimulation needed to induce anergy or to induce/expand Treg cells are different. In this context, tolerogenic capacity may even be related to DC longevity, since the time available to provide the appropriate signals to expand a limited pool of Treg cells may also be crucial [47].

13.3.1.4 Tolerogenic DCs: Intrinsic Capacity?

In a recent study, Farquhar et al. have demonstrated that, in the definition of tolerogenic DCs, the responding T cell is equally important. Whilst Dby (male) antigen can induce antigen-specific tolerance in female mice of an $H\text{-}2^k$ background, the

same antigen is immunogenic in H-2^b recipients [48]. The use of F1 (H-2^k × H-2^b) DCs did not affect the outcome, suggesting that under certain circumstances it is not the mode of antigen presentation, but the intrinsic properties of the responder T-cells that determine the outcome of the integrated response.

13.3.2 In Vitro Evidence for the Induction of Tolerogenic DCs

There are a variety of approaches to promote DC tolerogenic capacity for the induction of peripheral tolerance. Approaches to date include the use of pharmacologic agents such as immunosuppressive drugs, biological modification with anti-inflammatory cytokines, and the use of co-stimulation blockade and the transfection of genes that encode molecules that divert T cell responses to a regulatory phenotype (reviewed in [49]).

The pharmacological manipulation of DCs is an attractive approach to induce a stable and reliable phenotype for clinical use that will resist any in vivo modifying stimuli. Amongst the pharmacologic agents that have been studied for their modulatory effects on DC function are: corticosteroids [50], mycophenolate mofetil [51], calcineurin inhibitors [52], mammalian target of rapamycin (mTOR) inhibitors [53, 54], deoxyspergualin [55], Vitamin D3 [56], Vitamin E [57] and aspirin [58, 59]. For many of these agents, isolated studies on cytokine production, signalling pathways or phenotype have been conducted. Altogether, in vitro experiments with DCs modified with the above drugs, have demonstrated phenotypic characteristics of reduced co-stimulation (CD80 and CD86) increases in co-inhibitory ligands such as PD-L1 or ILT-3, impaired IL-12 and enhanced IL-10 production, hypo-responsiveness in responder T-cells, and the induction of anergic and Treg cells [45, 51, 52, 57, 60–70]. Amongst the best-characterised agents that have been tested on DCs are corticosteroids and Vitamin D3 and its analogues and more details about their effects are described below [50, 71–78].

Corticosteroids have a wide range of immunological effects, but most importantly in human monocytes and macrophages they downregulate cytokine genes (including IL-1, IL-6, TNF-α, IL-10 and MIP-1α (reviewed in [50]) at the transcriptional and post-transcriptional levels [79]. In addition, glucocorticoids regulate the secretory, bactericidal and phagocytic capacity of these cells [80]. Piemonti et al. demonstrated that increasing concentrations of glucocorticoids inhibit the differentiation of DCs from monocytes in the presence of IL-4 and GM-CSF [50]. In these experiments, dexamethasone was present throughout culture and prevented the loss of CD14 and CD16 and the expression of markers such as CD1a and the upregulation of CD86. The resultant cells were resistant to maturation with TNF-α or CD40L and were poor as immunostimulators. Other authors have also shown an increase in IL-10 production by dexamethasone treated human DCs [81], alongside inhibition of IL-12 [50] in monocyte-derived DCs but little effect on the maturation of Langerhans cells. The migratory capacity of human DCs is impaired by dexamethasone [82] and the production of

inflammatory chemokines CCL2, CCL3, CCL5 and CCL22 is suppressed [78]. A similar picture is observed with Vitamin D3 analogues with similar effects on the cytokine profile, maturational status and inhibition of migration, however, production of CCL2, CCL3 and CCL18 were increased following Vitamin D3 treatment [83, 84]. The combination of dexamethasone and Vitamin D3 treatment of DCs generates a phenotype which potently induces Treg cells [85].

Of the other pharmacological agents mentioned, both aspirin and rapamycin have significant effects on maturation and function of DCs. The other agents are neutral as regards differentiation, antigen uptake, phenotype and cytokine production (reviewed in [86]). Cyclosporine A is inhibitory to CCR7 and CXCR4 production [87]. A common feature of most of these agents, including aspirin, corticosteroids, Vitamin D3 and E, is their inhibition of NF-κB activation [57, 88, 89].

The genetic engineering of DCs is an area that has received considerable interest, although there are significant safety concerns about the use of therapeutic genetically modified cells for clinical use. Rather than the use of biological agents, the modulation of DCs to induce constitutive expression of immunoregulatory molecules to promote tolerance has been attempted. Most of the literature regarding transfected DC is with respect to murine models, however, human DCs have been successfully transduced with molecules such as Fas L or CTLA-4KDEL. In these studies, transduced human DCs are capable of inhibiting antigen-specific responses [90, 91]. The encouraging results with CTLA-4KDEL transduced human DCs, suggest that similar approaches piloted using mouse DCs, such as transduction with constructs encoding IL-10, TGFβ, TRAIL and IL-4, may have potential in human therapeutic applications [60, 92, 93].

Along similar lines, the ability of DCs to phagocytose double stranded oligodeoxyribonucleotides (dsODN) encoding decoy NF-κB binding sites, which inhibit nuclear translocation of NF-κB has been demonstrated in the mouse [94], whilst in human pDCs specific CPG oligonucleotide sequences have been shown to have tolerizing effects [95].

Whilst a multiplicity of approaches exists, the need for rapid generation of clinical grade cells for clinical application makes the use of therapeutic compounds that are already licensed for human administration an attractive option.

In summary, T cell unresponsiveness and/or expansion/induction of Tregs may be achieved through in vitro manipulation of DCs. In the next section, we will present evidence for the in vivo effect of tolerogenic DCs with respect to transplantation tolerance.

13.4 In Vivo Models of Transplant Rejection/Tolerance

A considerable component of transplantation biology has been described using rodent (mouse and rat) vascularised allograft rejection as the end point read-out; these include heart [96–99], kidney [100, 101] and pancreatic islet cell transplants [102–104].

The use of tolerogenic DCs in vivo to achieve transplantation tolerance has been demonstrated, particularly when combined with immunoregulatory agents which interfere with the inflammatory milieu. Immunoregulatory agents used in mouse transplantation models include CTLA4-Ig, IL-10 and TGF-β, anti-CD40 antibody (MR1) and anti-CD4/8 antibodies [105–107]. Despite the successes in these and other models (e.g. DCs treated with Vitamin D, aspirin, RelB silencing (reviewed in [49]) significant difficulty has arisen in achieving the same results in more stringent models (reviewed in [108]). Recently, Farquhar et al. [48] have demonstrated that LPS, TGF-β, IL-10 or Vitamin D treatment of DCs can induce dominant tolerance in one mouse strain (H-2^k) to Dby, but fail to do so in mice of an H-2^b background, using autologous or F1 DCs. This raises the possibility that the ability of tolerogenic DCs to induce tolerance depends on the stringency of the model.

The use of skin grafts is often considered to be one of the most stringent models to study allogeneic transplantation tolerance [109]. Skin grafts continue to be used because they are a potent and convenient tool in which to study the processes involved in the immunological response to engrafted tissues. Both antigen-specific and non-specific responses to skin allografts occur, but it is the antigen-specific T cell responses that determine the ultimate fate of the transplanted skin [110]. Whilst minor histo incompatible heart grafts are rejected more slowly than fully allogeneic transplants, there is no difference in the speed of rejection between these types of mismatches in skin grafts [111]. Skin allografts may be rejected by either $CD4^+$ or $CD8^+$ T cells alone at any degree of antigenic mismatch [112]. Accordingly, approaches that induce tolerance to vascular grafts fail to work in skin, presumably due to the LCs, DCs that predominate in this transplanted tissue [113].

From the earlier description, it is clear that DCs treated with immunomodulating drugs in vitro are maturation resistant and have the capacity to induce antigen-specific tolerance via a number of mechanisms [9, 45, 56, 59, 114]. However, when tolerogenic DCs are injected in vivo, there remains the risk that DCs lose their tolerogenic capacity. Whether the major risk is DC maturation due to the inflammatory milieu of the post-operative state or intercurrent infection or any other mechanisms such as donor DCs processing and presentation by recipient DCs will be discussed later.

In Sects.13.5 and 13.6, we will summarise the limitations of the use of tolerogenic DCs, particularly donor DCs and we conclude this review by showing evidence for an alternative strategy to target DCs directly in vivo to induce transplantation tolerance.

13.5 Inherent Limitations in the Use of Tolerogenic Dendritic Cells to Induce Peripheral Tolerance

13.5.1 Cross-Priming Versus Cross-Tolerance

The importance of donor DCs in transplantation was highlighted by the original passenger leucocyte experiments, which demonstrated that direct pathway-mediated cellular rejection was amplified by the presence of donor-derived DCs [100]. However, it is also evident that in some models of transplantation tolerance, such as spontaneous allograft acceptance or induction by donor-specific transfusion (DST), the presence of donor tissue-derived DCs is also necessary to achieve stable long-term engraftment [115, 116]. One possible interpretation is that whilst donor DCs are indeed an important source of intact alloantigen for direct pathway activation, they may also provide a source of donor antigen in the draining lymph node for the stimulation of Treg cells with indirect allospecificity (reviewed in [117]). Support for this concept is provided by experiments using the Y-Ae monoclonal antibody (specific for the H-2-A^b-Eα complex) to monitor the processing and presentation of H-2E in vivo. When H-2E-bearing DCs were injected into H-$2A^b$ recipients, within 2 days most of the recipient DCs in the draining lymph node expressed the Y-Ae epitope. The number of donor cells in the lymph node was significantly smaller than the number of recipient DCs that had processed the donor H-2E molecule [35]. This result implies that, when migratory donor DCs die, upon reaching the lymph node, they are phagocytosed and processed by resident recipient DCs. However, these experiments did not elucidate if cross-presentation by DCs leads to immunity (cross-priming) or specific unresponsiveness (cross-tolerance). Taking this one step further, if graft-derived donor DCs can be presented by recipient APCs, could this not also be the fate of donor-derived DCs adoptively transferred for therapeutic benefit, in which case the same question of cross-priming versus cross-tolerance arises.

The importance of cross-presentation as an antigen processing and presentation pathway in immunity is provided by significant experimental evidence. In this context, the processing of apoptotic cells by DCs results in the cross-presentation and cross-priming of anti-tumour CTL responses and consequently, augmented tumour immunogenicity [118]. In early experiments, examining the nature of immune responses to minor histocompatibility antigens (mH), a responder mouse expressing both H-2^b and H-2^d alleles primed in vivo with cells of H-2^b origin, but differing in mH antigens, demonstrated vigorous secondary responses to mH antigens restricted by both H-2^b and H-2^d in vitro, due to the presence of cross-primed $CD4^+$ T-cells [119]. This pathway of antigen presentation is finely balanced between immunity and tolerance. Immature DCs exposed to apoptotic cells demonstrate impaired LPS-induced maturation and migration to lymph nodes, and via a TGFβ-dependent mechanism expand $FoxP3^+$ Tregs [120] and can prolong allograft survival in mice [121]. More recently, Divito et al. [122] demonstrated that in vivo

targeting with apoptotic cells can ameliorate chronic allograft vasculopathy and downregulate indirect allospecific responses via quiescent DCs. The same group has demonstrated that vitamin D3 DCs, that are maturation resistant, are capable of inducing transplantation tolerance across a full allomismatch, however, they demonstrate rapid re-processing and re-presentation of the injected DCs, such that the major effect was dependent on the DCs as a source of antigen rather than tolerogenicity [123].

In our own experiments, we have demonstrated that DCs with tolerogenic capacity in vitro, fail to induce prolongation of skin allograft survival in vivo. More importantly, they may have sensitised the recipient. In these experiments LPS-matured BM-derived DCs were used following treatment with dexamethasone and Vitamin D3. This combination of drugs rendered these DCs tolerogenic as they were unable to induce alloantigen and antigen-specific responses in vitro. However, when injected in vivo, these drug-modified DCs appeared to have a limited life span as they were not detected 3 days following adoptive transfer. Interestingly, in parallel to their disappearance, processed alloantigen capable of stimulating alloantigen-specific T cells was evident. Our data suggest that drug-treated allogeneic DCs are rapidly removed from the host following adoptive transfer, presumably by host DCs which process and present alloantigen indirectly on self-MHC molecules. Although other cell types may be involved in this function (monocytes appear to degrade MHC proteins completely [124], B-cells appear to be relatively inefficient at cross-presentation [125]), it is the DCs that have a unique capacity to efficiently cross-present antigen on MHC class I [126, 127]. This alloantigen may then be available for several days to sensitise the recipient, irrespective of the treatment that the donor-derived DCs have received in vitro. Although in both our study and in Morelli's publication, donor-derived tolerogenic DCs are processed and alloantigens presented by endogenous DCs, different outcomes are observed. The reasons for this are under active investigation, one possible explanation being a difference in preparation of tolerogenic DCs and another is that additional therapies are always necessary to induce transplant survival when DCs are used to induce tolerance. Furthermore, the context of a transplantation-related inflammatory response or concurrent infection make an effector response all the more likely during this time frame [128]. The processing and presentation of injected DCs is likely to be accelerated if NK-mediated killing of the injected cells occurs, as with any significant MHC mismatch [123, 129]. The role played by NK cells in the killing of donor DCs and favouring indirect presentation has recently been formally demonstrated [123, 130]. It is, therefore, possible that, if drug-treated recipient-derived DCs pulsed with alloantigens, rather than donor alloantigen bearing DCs, are used, a different outcome could be achieved, resulting in the amplification of Tregs with indirect allospecificities [131].

13.5.2 The Risk of MHC Transfer

Donor antigen processing and presentation is not the only route of alloantigen acquisition by recipient DCs, since they may acquire intact donor MHC molecules by cell–cell surface transfer. We have clearly demonstrated the cell–cell transfer of intact MHC molecules between DCs both in vitro and in vivo [132]. Acquired MHC molecules are recognised by both $CD4^+$ and $CD8^+$ T cells suggesting that transferred MHC molecules remain intact, in keeping with observations by Knight et al. [133]. At the same time we have been able to demonstrate the uni-directional transfer of MHC between endothelial cells and DCs [132]. Our preliminary data suggest that MHC class I can be transferred between donor and recipient DCs in vivo in a skin transplant setting leading to recipient DCs acquiring intact donor MHC molecules. In addition, it has been shown that MHC class II is bi-directionally transferred between donor and recipient DCs in vascularised kidney and heart allografts [134]. We have proposed that the capacity of recipient DCs to acquire donor MHC molecules which are presented directly to recipient T cells, represents a third pathway of allorecognition, the semi-direct pathway [132] (reviewed in [135, 136]).

The semi-direct pathway may help to explain the "four cell problem" or "unlinked help" by which $CD8^+$ T cells, activated by donor DCs directly, receive help from $CD4^+$ T cells that have been primed by recipient DCs indirectly. Recipient DC that acquired intact donor MHC class I molecules via the semi-direct pathway can simultaneously activate $CD8^+$ T cells via the direct pathway and $CD4^+$ T cells recognising donor MHC peptide presented by recipient MHC molecules indirectly. Indeed, $CD4^+$ T cells with indirect anti-donor specificity have been able to amplify a direct anti-donor $CD8^+$ effector response [137]. Finally, in the context of tolerance, the acquisition of intact donor MHC molecules can explain how indirect pathway $CD4^+$ Treg cells can regulate $CD8^+$ effector T cells with direct allospecificity, as previously published [138].

The semi-direct pathway can also explain the apparent discrepancies in the observed efficiency of *cis* and *trans* co-stimulation. There is evidence that, when a single DC provides antigen and co-stimulation (*in-cis*) that this is more efficient than when a by-stander DC provides the co-stimulation (*in-trans*) [139]. However, others have shown that MHC class $II^{-/-}$ recipients could reject co-stimulation deficient grafts as rapidly as wild-type grafts [140]. In this setting the co-stimulation comes from bystander DC derived from the recipient and the MHC from the graft. It is possible that this represents efficient semi-direct presentation whereby endothelial-derived MHC molecules from the donor are acquired by the recipient DCs, that are co-stimulation replete, and are able to provide efficient *in-cis* co-stimulation.

Finally, it has been shown that DCs are capable of acquiring MHC class I and II molecules from both live and dead DCs in vitro and in vivo and exosomes secreted by DCs can also contribute to the source of antigen for transfer in the context of an allogeneic transplant [132, 141–143], as discussed in detail later.

Altogether, the apparent efficiency of the semi-direct pathway in vivo creates the problem that injected donor DCs, irrespective of their treatment, are a source of alloantigen that may be acquired by host DCs, which are, in turn, susceptible to maturation signals. This may overcome the potential benefit of DC cellular therapy.

13.5.3 Exosomes as a Source of Alloantigens

As mentioned above, exosomes derived from donor DCs may provide another way to transfer alloantigens to recipient DCs. Montecalvo et al. [144] have shown that donor-derived exosomes are taken up and presented by the recipient DCs which are recognised by direct specific $CD4^+$ T cells. In contrast, it has also been shown that in a transplant setting adoptive transfer of alloantigen rich exosomes renders recipient animals tolerant to a subsequent allograft [145]. The immunomodulatory properties of exosomes has been shown in other systems, for example, placenta-derived exosomes expressing FasL and PD-L1 downregulate T cell activation, neoplasm-derived exosomes, present in the sera of cancer patients, suppress T cell signalling in vitro and lastly intestinal epithelial cells release exosomes carrying food-derived peptides that could play a role in oral tolerance. As exosomes fail to stimulate T cells unless they interact with DCs, it is possible that presentation of suppressive factors (such as co-inhibitory ligands PD-L1 or ILT-3) expressed by exosomes derived from drug modified tolerogenic donor DCs by recipient DCs may represent a way in which antigen-specific T cells are switched off without the need for intact cells. More recently, CD73 has been shown to be expressed by DCs, and is upregulated by TGF-β [146]. Exosomes derived from cancer cells expressing CD73 and CD39 have been shown to directly suppress T cell function [147]. These two molecules are ectoenzymes capable of converting adenosine triphosphate (ATP) into monophosphate (AMP), then adenosine, which in turn is anti-inflammatory [148, 149]. As DCs produce exosomes constitutively, it could be suggested that if molecules such as CD73 are present on all exosomes, this could explain an anti-inflammatory effect.

In conclusion, we still do not know whether exosomes are a component of transplant rejection or whether they can be tools to induce transplantation tolerance. From the data available so far, it is clear that this depends very much on the array of molecules that the exosomes express, whether they are amplifying the alloimmune response or whether they can induce tolerance. More results need to be accumulated before reaching any conclusion.

13.6 Targeted Delivery of Alloantigen to DCs

With the above limitations in mind, we sought to determine if cross-presentation of allogeneic MHC molecules could be harnessed in a more productive way to induce antigen-specific tolerance by antibody targeted delivery of alloantigen to cDCs in the resting state. Finkelman et al. have previously demonstrated that host DCs can be targeted in vivo and that their state of activation determines whether the resultant immune response is of a stimulatory or tolerogenic nature [150]. One question that arises when thinking about this as a strategy for inducing tolerance in vivo, is which DC subsets should be targeted? As described earlier, DCs are not a homogeneous population, so would targeting just one or more DC subsets be advantageous? cDCs in the mouse have been divided into $CD8\alpha^+CD205^+$ and $CD4^+DCIR2^+$ (DC inhibitory receptor-2) subsets. These subsets have different capacity to direct the differentiation of T cells, with $CD205^+$ DCs inducing IFN-γ and Th1 responses and $CD4^+DCIR2^+$ DCs inducing Th2 responses. Furthermore, $CD8\alpha^+CD205^+$ DCs are better in cross-presenting and activating $CD8^+$ T cells while $CD4^+DCIR2^+$ DCs preferentially activate $CD4^+$ T cells although they can both equally acquire MHC class I molecules in vitro [141]. By delivering antigen specifically to these two subsets of cDCs via anti-DEC-205 (targeted at CD205) or anti-33D1 (targeted at DCIR2) antibody, effective presentation of antigen by MHC class I and class II molecules, respectively, occurred. Antigen-specific T cells became activated, proliferated and were deleted following interaction with DC and targeted antigen. Yamazaki et al. have extended further this analysis by looking at the effect of these subpopulations in the expansion/induction of Tregs by using in vivo DC targeting strategies. They demonstrated that $CD8\alpha^+CD205^+$ DCs produce TGF-β and induce Tregs while the $CD4^+DCIR2^+$ DCs are better at expanding natural Tregs. Although the $CD4^+DCIR2^+$ DCs subset can induce Tregs if exogenous TGF-β is provided.

On the basis of these reports, we decided to target $CD4^+$ cDCs with either a single MHC class I-derived allopeptide or the complete MHC class I molecule ($H\text{-}2K^d$) and look at transplantation tolerance induction in vivo [151]. Targeting this alloantigen to the $CD4^+$ $DCIR2^+$ DCs resulted in almost complete depletion of alloantigen-specific $CD4^+$ T cells. Moreover, among the remaining $CD4^+$ T cells there was an increased percentage of $CD25^+FoxP3^+$ expressing T cells. However, in this model, rejection due to $CD8^+$ mediated direct pathway responses occurred and long-term graft survival was only achieved by inhibiting the $CD8^+$ T cells. Targeting one DC subset with alloantigen does not, therefore, induce a state of dominant tolerance. An alternative strategy, such as combined DC subset targeting (DCIR2 and CD205 expressing DCs) may preferentially deplete additional effector T cells and targeting the $CD8\alpha^+CD205^+$ DCs in particular may additionally expand $CD4^+CD25^+FoxP3^+$ from na $CD4^+CD25^-FoxP3^-$ T cells.

More recently we have extended our study to target the same alloantigen to another population of DC, the plasmacytoid DCs (pDCs). Whilst pDCs are known to have a key role in a number of immune-mediated diseases such as psoriasis,

tumours and infections, relatively few studies have focused on the stimulatory capacity of pDCs in the context of inducing or modulating allogeneic responses in the transplantation setting. An emerging theme is that mature pDCs can induce Treg cells from na T cells both in vitro [152] and in vivo [153], along with an ability to convert na $CD4^+$ T cells into Treg cells [154]. The tolerogenic potential of pDCs has also been demonstrated by Ochando et al. [155] who showed that recipient origin pDCs are the main donor alloantigen presenting APCs in a murine model of heart transplantation tolerance.

Our findings show that, although pDCs have a limited capacity to induce de novo Tregs both in vitro and by targeting alloantigen to pDCs in vivo, they may be more effective at inducing the expansion of antigen-specific naturally occurring Tregs. We are currently investigating this capacity of pDCs further by combining targeted delivery of alloantigen with a maturation signal (CpG peptide) to pDCs in vivo, as a strategy to achieve regulation of antigen-specific T cell responses through expansion of allospecific Tregs.

13.7 Conclusions

It is clear that DCs with a reliable in vitro tolerogenic phenotype can be generated in a reproducible manner, however, in vivo, these cells are subject to maturational stimuli, independently of whether they are of donor or recipient origin. Furthermore, processing and presentation of donor tolerogenic DCs by recipient APCs or MHC transfer, are all mechanisms that may undermine their tolerogenic potential. However, the delivery of antigen to particular DCs subsets in vivo, by-passing the need for cellular negative vaccination, may promote mechanisms that favour tolerance while avoiding the risks of sensitisation due to cross-priming or MHC transfer.

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Chapter 14
Strategies for the Induction of Tolerance with Monoclonal Antibodies

Luis Graca

Abstract The introduction in the organism of tissues expressing foreign genes—ranging from major histocompatibility antigens to the products of gene therapy—have in common their ability to elicit protective immune responses leading to their rejection. Different strategies have been proposed to overcome immune rejection. Monoclonal antibodies, targeting molecules involved in the molecular events required for T cell activation, offer the promise of resetting the immune system toward tolerance without compromising overall immune competence. The mechanisms leading to immune tolerance rely not only on the induction of regulatory T cells, but also on the elimination of aggressive clones, and the triggering of specific gene expression programs that contribute to self-defense of the target tissue.

14.1 Introduction

Over thousands of years of coexistence with infectious agents with short life cycles, the immune system has evolved mechanisms able to detect small molecular differences between self and nonself in order to eliminate potential pathogens. This unique ability to discriminate small molecular changes to the normal composition of the organism became critical, not only as a defense mechanism, but also as a major hurdle for the therapeutic introduction of cells, tissues, or even immunogenic molecules into the human body.

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In fact, the transplantation of cells or tissues from unrelated donors, whether conventional allografts or derived from stem cells, triggers an immune response leading to the rejection of the graft unless a therapeutic strategy is used to modulate that immune response. Similarly, the correction of genetic diseases by therapeutically induced expression of the defective gene (gene therapy), or simply by providing the recombinant protein to replace the product encoded by that defective gene, can also elicit an immune response. This is the case, for instance, for patients with severe hemophilia treated with recombinant clotting factors. About one-fourth of those patients treated with recombinant factor VIII (FVIII) generate immunoglobulins, known as inhibitors, that target the therapeutic protein—perceived in that context as a non-self molecule—thus inhibiting its biological function [1]. Immunogenicity of therapeutic molecules can also be a problem at a time when biological drugs, such as monoclonal antibodies (MAbs), are increasingly used in the clinic. Even fully human MAbs bear non-self domains within their unique antigen-binding regions that can elicit the generation of anti-idiotypic antibodies, interfering with their therapeutic function [2].

In general, established therapeutic strategies to avoid undesirable immune responses, such as the ones leading to transplant rejection, rely on the functional amputation of the immune system as a whole with long-term usage of immunosuppressive drugs. These therapeutic regimens, frequently based on a combination of several drugs, are increasingly effective in preventing the unwanted immune response (e.g. transplant rejection), and will also inflict a number of undesirable, life-threatening adverse effects. As a consequence, a major objective of immunologists has been the development of therapeutic strategies able to prevent immune responses targeting a number of defined antigens without penalizing the immune system as a whole, something that has become known as immune tolerance.

MAbs have become successful drugs, for many different diseases, due to their ability to specifically target given molecules on the cell surface. Depending on their idiotype, the therapeutic MAbs can trigger effector mechanisms leading to the killing of the target cell or can simply block molecular interactions involving the target molecule.

The first MAbs routinely used to prevent unwanted immune responses in clinical transplantation had the main purpose of eliminating T cell populations. Such a strategy for achieving immunosuppression by reducing the number of T cells in transplant patients was not novel, as shown by old experimental methods such as the collection of lymph from the thoracic duct with a catheter or the use of polyclonal anti-lymphocyte sera [3, 4]. The specificity of MAbs, however, allowed a greater control over the target populations being depleted. Such is the case for CD3, CD25, or CD52 MAbs. In fact, the anti-CD3 MAb OKT3 (muroMAb) was the first MAb licensed for prevention of rejection episodes [5]. However, its immunogenicity associated with the triggering of a cytokine release syndrome has limited its use. More recently, targeting of CD52 (the most abundant antigen on the T cell surface) with CAMPATH-1H (alemtuzuMAb) was shown to induce T cell depletion leading to prevention of graft rejection with minimization of

maintenance immunosuppressive drugs [6–8]. The targeting of CD25—the α-chain of the interleukin-2 receptor (IL-2R)—with a MAb, offered the prospect of selective elimination of activated T cells [9]. However, T cells with immune regulatory function (Treg cells) can also be eliminated following this treatment [7], making such a strategy counterproductive for the establishment of tolerance.

In addition to the usefulness of MAbs as immunosuppressive agents acting by eliminating specific lymphocyte populations, there has been growing evidence supporting the use of MAbs for the induction of immune tolerance by modulating lymphocyte function [10].

14.2 Non-Depleting Monoclonal Antibodies as Tolerance-Inducing Reagents

It is now more than two decades since the initial demonstration that a brief treatment with MAbs can lead to long-term transplantation tolerance [7, 11, 12]. However, the mechanisms by which tolerance is induced and maintained are not yet fully understood. It has become clear, however, that antibody-induced tolerance can lead to both deletion of some alloreactive clones and Treg cell induction and/or expansion [13, 14].

In the initial attempts to induce peripheral tolerance with MAbs, depleting anti-CD4 MAbs were used to induce tolerance in mice to foreign immunoglobulins [11, 12]. It was later shown, however, that depletion of $CD4^+$ cells was not critical for tolerance induction, as similar results were found using F(ab')2 fragments [15, 16], non-depleting isotypes [17] or non-depleting doses of synergistic pairs of anti-CD4 MAbs [18]. A short treatment with non-depleting anti-CD4 MAbs was also shown to lead to long-term acceptance of skin grafts differing in multiple minor histocompatibility (mH) antigens [17], even in presensitized recipients [19]. Similar results were also obtained for heart grafts differing across MHC barriers [20, 21] or concordant xenografts [20]. The treated animals accepted the transplanted tissues indefinitely without the need for immunosuppression, and remained fully competent to reject unrelated (third-party) grafts. Remarkably, the tolerance-inducing treatment can be effective in animals where the entire T cell repertoire comprises alloreactive cells, namely in TCR-transgenic mice deficient in one of the *RAG* genes, transplanted with grafts expressing the target antigen [22]. All of the above clearly show that the antibody treatment can render the immune system tolerant to antigens of the transplanted tissue and not simply immunosuppressed.

However, CD4 is not the only target for tolerogenic MAbs. It soon became clear that several other MAb could lead to immune tolerance (Fig. 14.1). These include MAbs targeting T cell co-receptors (CD3, CD4, CD45); targeting co-stimulation (CD154 or CD40L, CD28, PD-1, ICOS); and targeting adhesion molecules (LFA-1, ICAM-1).

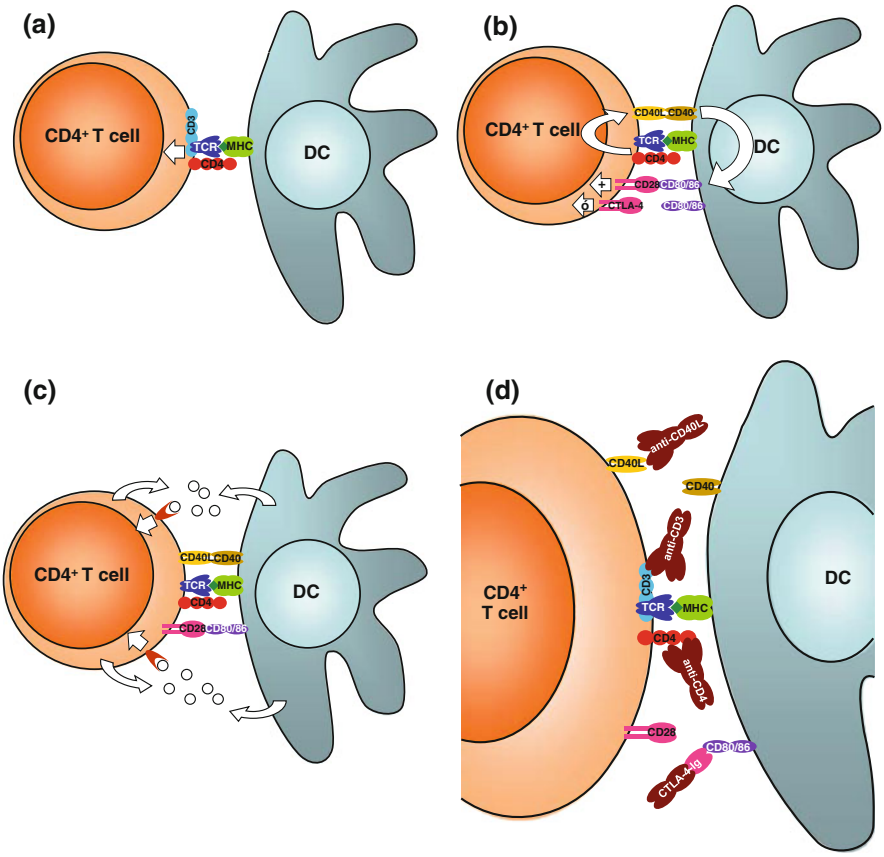

Fig. 14.1 Tolerance induction with monoclonal antibodies interfering with T cell activation. **a** Signal 1. The events leading to T cell activation start with TCR recognition of the appropriate antigen presented in the context of an MHC molecule. CD4 participates in this process, and the molecular complex CD3 is important for the signal transduction. **b** Signal 2. A consequence of signal 1 is the expression of CD40L by the T cell. The engagement of CD40L with CD40 drives the expression of the co-stimulatory molecules CD80 and CD86 by the DC. CD80 and CD86 bind CD28 on the T cell delivering potent stimulatory signals to the T cell. Activated T cells start expressing CTLA-4. As this molecule binds CD80 and CD86 with greater affinity than CD28, CTLA-4 contributes to the termination of the positive stimuli delivered by CD28. In addition, CTLA-4 delivers inhibitory stimuli to the T cell. **c** Signal 3. The T cell produces a high affinity IL-2 receptor, as well as IL-2 essential for its own proliferation. In addition, the DC releases cytokines able to influence the functional specialization of the T cell. **d** Several MAbs have been shown to induce immune tolerance. These molecules include, among others, non-depleting MAbs targeting the co-receptor molecules CD3 and CD4 and co-stimulation blockade with anti-CD154 and CTLA4Ig

Co-stimulation blockade, in particular, has been especially attractive as a strategy to control pathological immune responses and eventually induce tolerance [23, 24]. The initial targets for co-stimulation blockade were the

CD28–CD80/CD86 pathway, blocked with CTLA4Ig; and the CD40–CD40 ligand (CD154) pathway. Anti-CD154 MAbs were shown to be able to prevent disease in animal models of autoimmunity, and to achieve long-term transplantation tolerance in mice, rats, and non-human primates [25, 26]. Unfortunately, the presence of CD154 on human platelets has prevented the translation of these preclinical results into clinical treatments, due to the risk of thromboembolic complications [27]. Alternative approaches, to circumvent such adverse events, are being pursued, namely through the targeting of CD40 with MAbs lacking (or with minimal) agonist effects [28].

CTLA4Ig (abatacept) is, so far, the only drug blocking co-stimulation to be licenced for clinical use [29]. In transplantation, data on CTLA4Ig was not as impressive as with anti-CD154: CTLA4Ig could only achieve long-term transplant survival when given continuously or in combination with anti-CD154 [30, 31]. However, the low efficacy of CTLA4Ig in transplantation has been addressed through the design of a modified molecule named belatacept (or LEA29Y), containing a difference in two amino acids in the extracellular CTLA-4 domain, thus conferring greater affinity for CD80 and CD86 [32]. Phase II clinical trials in renal transplantation revealed comparable efficacy in reducing graft rejection as the standard immunosuppressive regimens but with a significant reduction of nephrotoxicity [33].

An additional member of the co-stimulation family is ICOS (inducible co-stimulator), which is induced shortly after T cell activation, and binds B7h, playing a critical role in germinal center reactions [34–37]. Mice deficient in ICOS, or treated with anti-B7h MAbs, were protected from collagen-induced arthritis [38]. In addition, anti-ICOS was also reported to have some immunosuppressive effects [39, 40]. An interesting report has shown that in a murine model of hemophilia A, correction of the genetic defect with a factor VIII expressing plasmid could evade the immune response following treatment with anti-ICOS MAb [41], suggesting that tolerance to FVIII could be achieved following transient ICOS blockade.

14.3 Dominant Tolerance

In experimental conditions where transplantation tolerance is induced with MAbs targeting co-receptors (non-depleting anti-CD4 and anti-CD8) or co-stimulation blockade (non-depleting anti-CD154) the tolerant state is robust enough to resist the adoptive transfer of lymphocytes from a non-tolerant syngeneic donor [17, 42, 43]. This capacity to prevent transferred cells from mediating transplant rejection—known as "resistance"—is a distinctive characteristic of dominant tolerance. When tolerance is induced through mechanisms that rely predominantly in deletion of alloreactive cells (for instance in mixed chimerism), resistance is not observed [44]. It has been shown that "resistance" is a property conferred by $CD4^+$ T cells [42, 45, 46]. Furthermore, when non-tolerant cells are allowed to

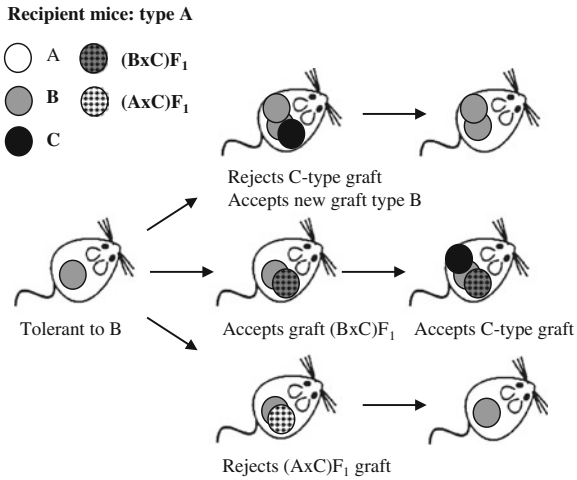

Fig. 14.2 Regulation through linked suppression. Mice rendered tolerant to an allograft, for instance following treatment with a MAb regimen leading to dominant tolerance based on Treg cells, will display linked suppression. These animals, tolerant to grafts of type-B, remain fully competent to reject third-party grafts of type C, even when transplanted simultaneously with grafts of type-B (*top*). However, they readily accept grafts from $(BxC)F_1$ donors, where both sets of antigens are present on the same cells (*middle*). Furthermore, after the acceptance of the $(BxC)F_1$ grafts the animals become tolerant of C-type grafts. This phenomenon of linked suppression is not observed with self-antigens, as tolerant mice reject $(AxC)F_1$ grafts (*bottom*)

coexist long enough with the T cells maintaining the tolerant state, they themselves acquire regulatory properties and start contributing to the maintenance of tolerance. This process has been named "infectious tolerance" [42, 45, 46].

An alternative feature of dominant tolerance has been known as "linked suppression" [47]: the property of tolerance to extend to additional allo-antigens when presented simultaneously (within the same tissue) as the tolerated antigens (Fig. 14.2). In other words, when tolerance is induced to a set of antigens, the immune system will also accept tissues that, in addition to the tolerated set of antigens, can express other alloantigens. However, if the second set of antigens is presented alone they are readily rejected [47]. This feature represents the imposition of tolerance over alloreactive cells that, on their own, would be able to reject a graft containing the second set of antigens. Remarkably, the self-antigens are not usually able to induce linked suppression. This probably represents the consequences of thymic negative selection, through which the frequency of T cells actively maintaining tolerance for the antigens presented by antigen presenting cells (APCs) may be below a critical threshold—we have termed this observation the blind-spot of Treg cells [48]. The exception to this rule is when transplantation tolerance is induced to fully mismatched grafts [44]. In this case, in a few occasions linked suppression can still be observed when an allogeneic graft is present

in tissues that also express self-MHC. We interpreted these observations as being most likely due to shared/cross-reactive alloantigens between the two allogeneic tissues that can be presented via self-MHC.

14.4 Treg Cells are Required for the Maintenance of Dominant Tolerance

Initial evidence that T cells were involved in immune regulation derive from observations made over three decades ago concerning neonatal transplantation tolerance, showing T cells could suppress responses to foreign proteins or allogeneic grafts, after adoptive transfer into irradiated secondary recipients [49, 50]. Furthermore, autoimmune manifestations developing in irradiated neonatally thymectomised animals could be prevented by the adoptive transfer of thymocytes or splenocytes from normal syngeneic donors [51–53]. The development of methods allowing specific depletion or sorting of T cell subsets enabled further characterization of the phenotype of the cells preventing the onset of autoimmune diseases or gut immunopathology upon adoptive transfer into susceptible animals. Sakaguchi and colleagues identified the regulatory capacity among the $CD5^+$ T cells [54], while in experimentally induced tolerance, regulatory activity was present among the $CD4^+$ T cells [55]. The $CD4^+$ T cells were further subdivided: first the regulatory activity was found to be within the $CD4^+CD45RC^{low}$ compartment in the rat or the $CD4^+CD45RB^{low}$ compartment in mice, and later within the $CD4^+CD25^+$ subpopulation [56–58]. Finally, the major subset involved in regulation was ascribed to T cells expressing the transcription factor Foxp3 (a large proportion of the $CD4^+CD25^+$ subset) [59–61].

It has been shown that several of the strategies leading to the induction of tolerance with the MAbs described in the previous section do so by inducing Treg cells [62, 63]. Furthermore, there is evidence that Treg cells maintaining transplantation tolerance can be found not only within the spleen and lymph nodes, but also infiltrating the tolerized allograft [64]. This observation suggests that part of the activity of Treg cells may be to impose a local state of immune privilege, as discussed below [65, 66].

The study of Treg cells in the maintenance of dominant tolerance had a significant boost following the identification of the transcription factor Foxp3 as a molecular marker of Treg cells, able to impose a regulatory phenotype on non-regulatory T cells [59–61]. It became possible to use a molecular marker allowing a distinction between Treg cells and activated T cells, since an acute infection or TCR stimulation does not appear to trigger Foxp3 expression in mice [67]. However, recent reports have suggested that unlike in mice, Foxp3 may be transiently expressed in human activated T cells [68]. It was also recently shown that natural killer T (NKT) cells can also acquire immunosuppressive function following induction of Foxp3 [69]. Although not all Treg cells express Foxp3 [70],

absence of Foxp3 compromises immune tolerance leading to severe autoimmunity both in animal models [71] and human patients [72]. Besides a natural population of $Foxp3^+$ Treg cells produced in the thymus [73], there is now evidence that non-regulatory $CD4^+$ lymphocytes can be converted into $Foxp3^+$ Tregs in peripheral tissues [74, 75].

In addition to the role of $Foxp3^+$ Treg cells in preventing autoimmunity, allergy, gut immunopathology and transplant rejection [76], it is now established that Treg-mediated suppression may be associated with deleterious effects, namely inhibition of anti-tumor immune responses or protective responses against pathogens [77, 78].

14.5 Therapeutic Conversion of Naïve T Cells into Treg Cells

The study of peripheral Treg cell conversion was greatly facilitated with TCR-transgenic RAG-deficient mice. Such animals are unable to produce a functional TCR from their endogenous genes. As a consequence, all T cells exclusively express the transgenic TCR. Thymic development in the absence of an appropriate ligand for the transgenic TCR results in a population of mature T cells without Foxp3 expressing Treg cells [79]. Therefore, it becomes possible to examine the conversion of such T cells into $Foxp3^+$ Treg cells, in the absence of a possible contamination due to the expansion of pre-existing Treg cells.

It was possible to document the de novo induction of Treg cells following in vitro antigenic stimulation by dendritic cells (DCs) in the presence of non-depleting anti-CD4 MAb [22], using TCR-transgenic $RAG^{-/-}$ mice specific for a given male antigen. Similarly, Treg cells from the same animals were converted in vivo following transplantation of male skin grafts onto female TCR-transgenic mice treated with tolerogenic anti-CD4 MAbs [22]. The same mouse strain was also used to demonstrate that in vivo exposure to the antigenic peptide or to an appropriate altered peptide ligand also leads to peripheral induction of Treg cells and dominant transplantation tolerance [63, 80]. Immature DCs or DCs treated with 1α,25-dihydroxyvitamin D_3 (a reagent that prevents subsequent DC maturation) when adoptively transferred into male-specific TCR-transgenic female mice equally lead to peripheral induction of $Foxp3^+$ Treg cells and dominant tolerance to male skin grafts [81]. The peripheral induction of Treg cells, both in vitro and in vivo, seems to require TGF-β as it is abrogated in the presence of neutralizing anti-TGF-β MAbs [22, 81].

TCR-transgenic $RAG^{-/-}$ mice specific to chicken ovalbumin (OVA) were also used to investigate extra-thymic conversion of $CD4^+$ T cells into Treg cells. It was shown that exogenous addition of TGF-β to T cell cultures in vitro led to induction of $Foxp3^+$ Treg cells [82]. Furthermore, the use of low concentration of OVA peptide for in vitro activation of $Foxp3^-$ T cells also resulted in induction of

Foxp3$^+$ T cells [83]. Interestingly, B cells were shown to be more efficient than DCs in driving Treg conversion, presumably by their inability to provide full co-stimulatory signals [83].

Oral tolerance, induced in mice by exposure to OVA in the drinking water, was also shown to lead to the conversion of TCR-transgenic OVA-specific T cells into Foxp3$^+$ Treg cells [84]. Of note, oral exposure to a potent NKT cell agonist —α-galactosylceramide—was also shown to lead to induction of Foxp3 expression by NKT cells in the gut [69]. This process was TGF-β-dependent, as no Foxp3 induction occurs in mice with NKT cells without a functional TGF-β receptor [69].

In addition, in vivo exposure of T cells to a low dose of persistent antigen also resulted in Foxp3$^+$ Treg induction [85]. Spontaneous conversion of non-regulatory T cells into Foxp3$^+$ Tregs was also claimed following adoptive transfer experiments of CD4$^+$CD25$^-$ T cells into congenic mice [86, 87]. However, in animals that are not TCR-transgenic RAG$^{-/-}$ it is always difficult to exclude a contribution of Treg expansion by some contaminating Foxp3$^+$CD25$^-$ cells [67].

A different population of Treg cells, named Tr1 cells, has been described [88]. These cells do not express Foxp3, are peripherally induced by antigenic stimulation in an IL-10 rich environment, and are characterized by IL-10 production. Although Foxp3$^+$ Treg cells are critical in preventing autoimmunity, Tr1 cells may become useful therapeutic tools for the suppression of immune pathology [89].

T cell anergy—a state in which T cells remain viable but unable to respond to optimal stimulation through both the TCR and costimulatory molecules—was also reported in vivo in several animal models under conditions quite similar to the ones leading to Treg induction. Following initial studies showing that T cell exposure to high doses of influenza virus haemaglutinin would lead to T cell anergy [90], it was shown that a similar state of T cell unresponsiveness could be achieved by antigen recognition in the absence of co-stimulation [91, 92], the use of altered peptide ligands [93, 94], or direct presentation by activated rat or human T cells which express MHC class II molecules [95, 96]. Furthermore, anergy was also documented following transplantation tolerance induced with anti-CD4 MAbs [97, 98], by the injection of cells expressing the self-superantigen Mls-1a in mice [99], by aqueous peptide antigen administration in mice [100], in double transgenic mice for a TCR and its surrogate antigen [101, 102], and in oral tolerance [103].

Given the current tools available for the identification of Treg cells [104] it would be relevant to revisit these experimental systems to address the contribution of Treg cell induction.

14.6 Suboptimal Activation for the Peripheral Induction of Treg Cells

It is now accepted that thymic generation of Treg cells requires recognition of antigen [79], with this requirement shaping the Treg cell TCR repertoire toward self-recognition [105]. However, it appears that when thymic recognition reaches a

certain threshold for thymocyte activation it results in the induction of apoptosis and negative selection [106]. The observations described in the previous section, together with knowledge of thymic Treg generation, led us to propose [74] that peripheral Treg induction probably mirrors the thymic events: if a T cell encounters antigen in an inflammatory environment supporting full activation it will commit toward an aggressive phenotype appropriate to the initiation of a protective immune response; if, on the other hand, a T cell interacts with the antigen in an environment conducive to suboptimal activation, it will differentiate toward a regulatory phenotype. Factors contributing to suboptimal activation—MAbs targeting molecules involved in the immune synapse, low concentration of the cognate peptide, altered peptide ligand, or immaturity of the DC—can, therefore, facilitate the conversion of naïve T cells into Foxp3$^+$ Treg cells. Our hypothesis is further supported by observations that mutations in T cell stimulatory components—such as Lck—may facilitate Treg cell development [107].

14.7 Tolerance and Tissue Self-defense

The anatomical location where Treg cells operate in vivo also requires further clarification. It has been reported that Treg cells can be isolated from secondary lymphoid organs such as lymph nodes and spleen, but Treg cells able to prevent transplant rejection can also be found infiltrating the tolerated transplant [64]. Remarkably, Treg cells accumulate preferentially within the tolerated graft (e.g., transplanted skin), but not within skin of host origin. It will be important, therefore, to clarify whether a local immune privileged site may be induced through the action of Treg cells. Such a notion is in keeping with studies on tumor evasion, suggesting that tumor infiltrating Treg cells may induce local protection against anti-tumor immunity and therefore promote immune escape [108].

A key protective gene in transplantation tolerance is hemoxygenase-1 (HO-1) [109]. HO-1 catalyzes heme degradation leading to the local production of equimolar amounts of carbon monoxide (CO), biliverdin, and free-iron which induces the expression of heavy-chain ferritin, an iron-binding protein. All three metabolites, resulting from heme degradation by HO-1, may have an immune protective effect [110].

Furthermore, the local immune response seems to be under tight control of local availability of specific essential amino acids [111]. The contribution of the tryptophan catabolizing enzyme indolamine 2,3-dioxygenase (IDO) to fetal tolerance was the first reported example of regulation by essential amino acid depletion [112, 113]. IDO was also implicated in the maintenance of tolerance following co-stimulation blockade by CTLA4Ig, leading to Treg cell induction [114, 115]. These observations were recently generalized with the finding that several other enzymes that catabolize essential amino acids are overexpressed under tolerogenic conditions, where the amino acid consumption prevents T cell proliferation and facilitates TGF-β-dependent Treg induction [111].

14.8 Contribution of Cell Death for Peripheral Immune Tolerance

Immune tolerance appears to require not only the induction of dominant regulatory mechanisms, such as Treg cells, but also the elimination of some T cells committed toward aggressive function. In fact, it has been known that efficient elimination of alloreactive clones can lead to transplantation tolerance, namely following a protocol promoting a state of mixed chimerism with transplantation of donor hematopoietic stem cells [116]. However, in antibody-induced peripheral tolerance following co-stimulation blockade, some degree of cell death seems to be required [117, 118]. In fact, when activation-induced cell death (AICD) is blocked, either by using a transgenic mouse strain resistant to apoptosis [117], or by using cyclosporin A [118], skin grafts transplanted under the cover of CTLA4Ig and anti-CD154 MAb are rejected. This rejection occurs despite the fact that Treg cells are induced under these conditions of co-stimulation blockade [42], but clearly are insufficient to prevent rejection in the absence of some degree of AICD of aggressive T cells.

It is also likely, although not yet formally demonstrated, that other tolerogenic MAbs such as anti-CD4, also require some degree of AICD in addition to Treg induction. A study has shown that tolerance induced with anti-CD4 is independent of the Fas (CD95) pathway [119]. Probably all tolerance-inducing strategies involving MAbs require AICD to eliminate some T cell clones that are committed toward an aggressive phenotype, together with functional inactivation (anergy) of some of the T cells, and also the induction of Treg cells.

14.9 Conclusion

In the last decade, the rate of adoption of MAbs in clinical practice is a demonstration of the potential of these therapeutic agents with exquisite specificity. However, the first MAbs in the clinic represent what will probably be regarded as a first generation of therapeutics that bring about, in general, the elimination of cells or the neutralization of molecular mediators (such as pro-inflammatory cytokines). The challenge of tolerance induction requires greater sophistication, as clearly the elimination of cellular subsets or molecular mediators will not lead to stable tolerance (although it may be sufficient to attain a “near tolerant” state that may be advantageous by allowing the reduction of conventional immunosuppression in transplantation [120]). Such sophistication will require the modulation of T cell function with the emergence of a regulatory population able to maintain the tolerant state. In this regard, MAbs that target molecules involved in the immune synapse leading to T cell activation offer great promise as a way of tipping the balance of the immune response toward tolerance.

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Chapter 15
Induction of Immunological Tolerance to Transgene Products

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Abstract Gene transfer holds the possibility of correcting difficult or impossible to treat disorders, ranging from inherited monogenic diseases to graft rejection in transplant surgery. However, one barrier to success in gene therapy is the immune response to the protein encoded by the transgene. Because the transgene product is often missing in the patient, they will have developed lymphocytes capable of generating an immune response to the therapeutic protein, thereby rendering treatment ineffective. Thus, finding a way to induce tolerance to the gene product is the focus of a considerable amount of research. This research often relies heavily on delivery of the transgene to anatomical sites of the body that are naturally immune privileged or potentially immune suppressive, such as the liver, central nervous system, eyes, and hematopoietic cells. The benefits of tolerance induction by gene transfer are not limited for using gene therapy as a protein replacement strategy but can be extended to any situation where an immune response is unfavorable, such as autoimmune disease. This chapter focuses on the different strategies used to induce tolerance to transgene products along with the practical applications and limitations of those strategies based on the physiology of each anatomical site targeted.

15.1 Introduction

During lymphocyte development, newly generated cells undergo a process of selection to eliminate effector cells capable of mounting an immune response against "self" antigens. For T cells, this occurs in the thymus in a process called

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"central tolerance" where T cells will be eliminated if their T cell receptor (TCR) either does not bind products of the major histocompatibility complex (MHC), binds self-MHC with too high an avidity, or binds a self-peptide: self-MHC complex. However, in order for an antigen to be selected against, it needs to be expressed in thymic epithelial cells, the cells responsible for presenting antigens to developing T cells. Since not every protein is necessary for thymic epithelial cell function, and would not, therefore, be expected to be expressed, the transcription factor AIRE directs the expression of a wide array of proteins in order to expose developing T cells to as many "self" proteins as possible. Thus, by the time T cells emerge from the thymus, a large proportion of potentially auto-reactive cells have been removed. Those that escape selection can be eliminated or inactivated via "peripheral tolerance" if they encounter self antigen in the absence of immune danger signals that usually indicate an infection or cell damage. Some autoreactive T cells may assume an alternative, regulatory phenotype (T regulatory cells or "Treg") in which they serve to dampen or prevent an immune response to self antigens. Development to this cell type can occur both in the thymus and in the periphery in the case of a T cell that has escaped central tolerance.

A similar selection process occurs in the bone marrow where B cells with auto-reactive B cell receptors undergo receptor editing to change their specificity or die by apoptosis if they are unable to create a non-self-reactive receptor. Furthermore, if an auto-reactive B cell escapes to the periphery it will still require help to produce antibody from $CD4^+$ T cells that recognize the cognate self-antigen. Given that auto-reactive T cells should have been removed by central or peripheral tolerance mechanisms, an autoantibody response is a rare event.

Because we are exposed to many exogenous antigens, which may not be derived from a pathogen, it is important that the immune system has evolved mechanisms to limit responses to novel antigens that enter the body in the absence of infection or other inflammatory signals. Nonetheless, if a person is lacking a functional gene for a protein, then that person may develop lymphocyte responses specific for a protein that would be considered "self" in healthy individuals. This is a particular problem in treatment of genetic diseases. For example, hemophilia A is an X-linked clotting disorder where a person is unable to produce a functional clotting factor (factor VIII, FVIII) due to a gene deletion or other mutation. Treatment of hemophilia A relies upon administration of exogenous recombinant FVIII protein [1]. Since FVIII has not been present during lymphocyte development it will not be recognized as "self" by the patient. Consequently, 20–30 % of patients mount a neutralizing antibody response against FVIII—rendering future treatment with exogenous FVIII ineffective. One proposed solution for hemophilia A has been the introduction of a functional gene for the clotting factor via gene therapy. But even in this context, vector-derived expression of FVIII is seen as foreign and an immune response can be directed against cells expressing the "foreign" transgene. Solutions to this problem are discussed in more detail below and largely drive research investigating tolerance induction to gene therapy products.

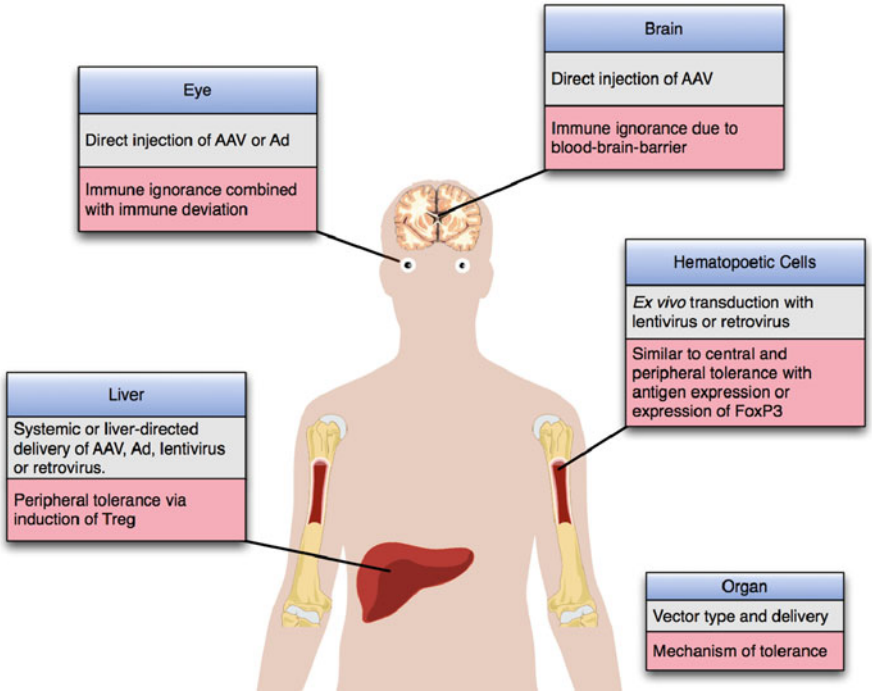

Fig. 15.1 Outline of sites currently known to achieve tolerance to gene products following delivery. For each site the most efficacious vector and route of administration are shown (*gray area*) as well as the specific mechanism of tolerance for each anatomical location (*red area*). Abbreviations: *AAV* Adeno-associated virus; *Ad* adenovirus; *Treg* T regulatory cell

The mechanisms of central and peripheral tolerance also influence tissue transplantation, as two individuals will only be tolerant of the own specific antigens, namely their HLA-associated antigens. Therefore, lymphocytes from a donor may respond to antigens of the recipient or vice versa. A considerable amount of work has been devoted to circumventing rejection of a transplant with immune suppressive regimens but also with novel techniques adapted from other fields—such as gene therapy.

Both the correction of genetic defects with gene therapy and transplantation of an allograft must overcome the challenge of introducing a foreign antigen into an immune competent individual without provoking an immune response, which stands as the major barrier to success in both fields. There are, however, certain sites of "immune privilege" in the body where immune responses are either suppressed, or the organ is isolated from the peripheral immune effectors (Fig. 15.1). These sites are attractive for gene therapy and transplantation due to the relative ease of introducing foreign antigens with minimal or reduced immune responses. One such site is the liver.

15.2 Gene Transfer to the Liver

The liver was first recognized as immunologically unique when it was discovered that successful liver transplants were possible between two MHC-mismatched pigs [2]. The tolerogenic nature of the liver most likely has to do with the fact that it is directly downstream of the gut blood flow; therefore, it receives large amounts of innocuous foreign antigens from food, ingested bacteria, and commensal bacteria [3, 4]. Indeed, rather than an immune response, the liver will often favor immune tolerance when presented with a foreign antigen [5]. "Tolerance" in the context of gene transfer is typically defined as the absence of an immune response to the introduced antigen, as if it were a "self" antigen. This can either arise via active suppression of an immune response or through immunological "ignorance", that is the lack of an immune response due to the antigen being inaccessible to immune effector cells. Finally, antigen presentation in the absence of costimulatory molecules, which may be the case in the context of limited inflammatory signals such as the hepatic microenvironment, can result in T cell tolerance.

Gene therapists have exploited this concept of induced tolerance for multiple disease models by directing transgene expression to the liver to circumvent adaptive immune responses to therapeutic proteins used to treat genetic diseases [6]. The specialized cells and lymphocytes incorporated into the microanatomy of the liver contribute to its tolerogenic nature. The liver is a major site of lymphocyte migration and houses up to 80 % of the body's macrophage population, known as Kupffer cells in this location [4]. Kupffer cells, along with liver sinusoidal endothelial cells (LSECs) and liver dendritic cells, present antigen in the context of suppressive cytokines such as IL-10 and TGFβ, which render effector T cells inactive/tolerogenic rather than releasing the typical pro-inflammatory cytokines which invoke an adaptive immune response [3, 7–9].

Nonetheless, immune responses in the liver do occur given the correct context, and this is certainly true for gene therapy. This has been demonstrated with attempts at gene therapy in the liver using adenovirus in both animals and humans. In preclinical studies, delivery of adenovirus evokes a strong innate immune response that limits transgene expression even after the removal of the endogenous viral genes [10, 11]. This results not only in poor and transient expression of the therapeutic protein but can have more dire outcomes as demonstrated in a clinical trial of adenoviral gene therapy where one patient died following treatment due to an overwhelming immune response to adenoviral vector [12]. This has provided much of the impetus to investigating less immunogenic vectors such as adeno-associated virus (AAV) and defining ways to circumvent the immune response to gene transfer [11, 13, 14]. AAV is still limited by the requirement that minimal level of expression must be reached to induce tolerance and by potential immune responses to capsid antigen—as demonstrated in a recent clinical trial where AAV use in humans was complicated by a CTL-mediated destruction of transduced hepatocytes [15–17].

The cells responsible for an immune response to gene transfer are most likely antigen presenting cells (APCs) such as Kupffer cells. The best evidence for this comes from studies where transgene expression has been eliminated in APCs and, at the same time, specifically targeted to hepatocytes. One way to achieve this has been the use of hepatocyte-specific promoters, which have improved long-term transgene production in adenoviral, AAV, and lentivirus, another common viral vector [18–21]. Another approach has been to include a microRNA (miRNA) sequence in the transgene that will target the mRNA for destruction in hematopoietic cells, thereby further limiting expression in APCs and improving tolerance induction [22].

Inducing tolerance to transgene products via gene transfer to the liver not only allows for stable expression of a therapeutic protein, but also induces tolerance to this "foreign" antigen in the periphery. For example, mice that were tolerized to human factor IX (hFIX) via liver-directed gene therapy with AAV, remained immunologically unresponsive to a subcutaneous challenge with hFIX in complete Freund's adjuvant (CFA) or after a second round of gene transfer with the more immunogenic adenoviral vector [15, 23, 24]. Similarly, mice lacking endogenous acid α-glucosidase (GAA), mimicking Pompe disease, failed to mount an immune response against i.v. recombinant GAA protein following tolerization with an AAV–GAA vector [25]. While this is encouraging for protein deficiency diseases such as hemophilia and lysosomal storage diseases, researchers have also extended these findings to other disease models. Lohse et al. used liver-specific expression of myelin basic protein (MBP) to suppress experimental autoimmune encephalitis (EAE), a mouse model for the human autoimmune disease multiple sclerosis, induced by peripheral challenge with MBP and adjuvant [26]. Importantly, this and other studies demonstrate that immune tolerance to a protein antigen expressed by hepatocytes and facilitated by Treg induction extends to other compartments of the body, thereby causing unresponsiveness to the antigen expressed in other organs such as the brain or muscle or introduced systemically [24, 25, 27].

The mechanism of how gene transfer to the liver suppresses a peripheral immune response was elucidated when investigators found an increase in antigen-specific $CD4^+CD25^+$ T cells in animals that had received AAV-mediated gene transfer to the liver [28]. Furthermore, T cells from tolerized mice failed to respond to antigen stimulation in vitro and in later experiments adoptive transfer of $CD4^+CD25^+$ cells suppressed antibody responses to mice challenged with antigen in CFA [28, 29]. However, if these cells were depleted of $CD4^+CD25^+$ T cells, the suppression was abrogated, providing evidence for a crucial role for these cells in the induction of tolerance following gene therapy [29]. The normal function of such Treg cells is to protect against aberrant immune responses, such as those seen in autoimmune diseases [30]. It is now clear that these cells are induced following liver gene therapy and are required for tolerance to the transgene product [31]. In some cases, additional mechanisms of T cell tolerance such as deletion of transgene product-specific T cells or T cell anergy may synergize with Treg-mediated suppression, while in other models, peripheral Treg induction may be sufficient to achieve antigen-specific

tolerance. Induced Treg are capable of suppressing antibody and T cell responses to the transgene product, thereby also limiting hepatic inflammation and immunotoxicity that may otherwise be caused by responses to the transgene product.

15.3 Gene Transfer to the Eye

Similar to the liver, the eye and brain were discovered to be sites of immune privilege during transplantation experiments. In a seminal study of transplantation, Peter Medawar established the immune response as responsible for graft rejection. Two exceptions were the anterior chamber of the eye and the brain, which were found to accept allogeneic transplants much better than other sites in the body [32]. It is now known that antigens in the eye produce a deviated immune response that suppresses typical Th1 and Th2 responses in a process termed anterior chamber associated immune deviation (ACAID) [33]. Again, this process involves the protolerogenic cytokines IL-10 and TGF-β, as well as both $CD4^+$ and $CD8^+$ Treg [33]. The eye also owes some of its immune privilege to its relative isolation from the rest of the body via a tight blood-tissue barrier as well as paucity of draining lymphatics, APCs, and MHC expression [33]. Therefore, the eye is an ideal place for gene therapy as it would be expected to avoid immune responses to the vector and the transgene product allowing for stable, long-term tolerance to the therapeutic protein. Indeed, preclinical studies showed successful AAV delivery to the subretinal epithelium with minimal inflammation usually only resulting from the physical trauma of the injection [34, 35]. One particular issue in gene therapy using popular viral vectors is a neutralizing antibody response against the virus capsid that prevents future treatments with the same viral vector [13]. Preclinical studies investigating AAV injections in the eye, however, have shown an absence of neutralizing antibodies to AAV at lower doses, allowing for supplementary treatment in the opposite eye [36]. This work led to the recent clinical successes in gene therapy of Leber's congenital amaurosis, an inherited disease causing blindness. In three separate clinical trials, not only were deleterious immune responses avoided, but all patients also experienced a significant improvement in visual function [37–39].

There are, however, distinct differences between immune deviation in the eye and immune tolerance in the liver. One study, comparing Ad and AAV delivery to the eye, found that antibodies are formed to both the viral vector and to the transgene [40]. These antibodies were non-neutralizing, the eye was protected from inflammation following peripheral challenge with the same vector, and subretinal delivery of either vector to the eye prevented a delayed type hypersensitivity reaction in response to intradermal challenge with the vector, indicating a deviated immune response, if not complete tolerance [40]. Furthermore, whereas, liver tolerance can protect peripheral sites from immune responses and allow for subsequent gene therapy even with more immunogenic methods, antigen expression in the eye fails to protect mice in the same manner [15, 23, 24, 26]. Mice given AAV in the eye are not

protected from experimental autoimmune uveoretinitis—where an immune response is directed at retinal antigens via a peripheral challenge—and are still susceptible to CTL elimination of lentivirus-transduced hepatocytes [41, 42]. It is likely that the immune privilege of the eye is distinct from that of the liver and may be best suited for traditional gene replacement approaches, as opposed to the broader immune tolerance opportunities offered by the liver.

15.4 Gene Transfer to the Brain

The brain is another site of the body that is often associated with immune privilege. The brain and spinal cord are separated from the rest of the body by the blood–brain barrier (BBB)—a network of tight endothelial junctions that prevents the normal exchange of fluid, solutes and cellular infiltrates that occurs in the periphery. And while the liver and eye clearly have active immune suppression/deviation abilities, the brain may owe more of its privilege to mere isolation and immune ignorance. This is positive for gene therapy as viral and transgene antigens would be less accessible to the immune cells and lymphatics that are common in the systemic circulation. Furthermore, neutralizing antibodies in the circulation that would normally inhibit viral transduction should be excluded by the BBB and would not be expected to negatively impact gene therapy in the brain. Preclinical studies of gene delivery in the brain have had mixed results depending on which vector is used and the timing of treatment. Adenovirus was uninhibited in the presence of peripheral neutralizing antibodies against Ad capsid [43, 44]. AAV, on the other hand, does elicit a humoral immune response, albeit an attenuated one, following intrastriatal injection that can inhibit expression from a second injection given within 2 weeks—indicating a lack of complete tolerance or ignorance [45]. In fact, peripheral immunity to the vector transgene product can clear transgene expression in the brain in a manner that may involve CTL, suggesting that the brain may not be entirely isolated from the rest of the body [46, 47].

15.5 Hematopoietic Stem Cell Gene Transfer and Tolerance

The concept of genetically modifying hematopoietic stem cells (HSCs) to induce tolerance derived from observations in transplantation studies where it was shown that generating mixed donor-host hematopoietic chimerism resulted in immunological tolerance to MHC proteins and other minor histocompatibility (mH) antigens [48, 49]. Although this strategy was effective at inducing tolerance [50], allogeneic bone marrow transplantation poses the risk of inducing graft versus host disease (GvHD) as well as the risk of engraftment failure. These risks prompted investigation into the use of gene therapy vectors to transduce autologous bone marrow cells as a way of inducing molecular chimerism [51]. From these initial studies, the

genetic modification of HSCs has now been utilized to induce tolerance in tissue transplantation, autoimmune disease, and gene correction of inherited diseases using a variety of different antigens and in multiple animal models and species (Table 15.1) [52–54].

Gene transfer to HSCs requires a vector capable of providing stable, long-term expression, and offers many distinct advantages over transfer to solid organs (Fig. 15.1). Ex vivo gene transfer allows for a higher percentage of genetically modified cells, generally requires less vector and avoids potential immunological complications from systemic delivery. Most gene transfer protocols for HSCs use either simple retroviral vectors or lentiviral-derived vectors, where the latter are capable of gene transfer to non-dividing cells, which requires less manipulation of the HSCs. Optimized gene transfer protocols have been established for each of these viral vectors, allowing for up to 80–90 % gene transfer efficiency. While beyond the scope of this chapter, it should, however, be remembered that such integrating vector systems pose a risk of insertional mutagenesis.

The ability of gene modified HSCs to induce tolerance is dependent on efficient engraftment, which often requires high doses of total body irradiation for myeloablation, whereas non-myeloablation conditioning using lower doses of radiation or chemicals such as busulfan have been shown to provide a state of hyporesponsiveness [53, 55]. There is some evidence that suggests that the ability of transduced HSCs to either induce a state of hyporesponsiveness or tolerance is dependent on the level of antigen expression [55]. Therefore, strategies that can improve gene transfer efficiency and engraftment will have a greater chance of inducing tolerance [52, 53]. The mechanism of tolerance from gene transfer to HSCs is currently under investigation and there is accumulating evidence that suggests that both central tolerance mechanisms [56] (thymic deletion from antigen presentation of dendritic cells) and peripheral tolerance (induction of Treg) contribute to tolerance.

Some of the earliest studies investigating gene transfer to HSCs were focused around the generation of molecular chimerism to promote transplantation tolerance both to allografts [55, 57, 58] and xenografts that express the αGAL carbohydrate moiety [59, 60]. To extend the applications of this approach, it was demonstrated that retroviral gene transfer of green fluorescent protein (GFP), a cytoplasmic protein, resulted in tolerance to GFP [61] and to skin grafts from GFP transgenic mice [62]. Extending on their studies of gene transfer to B cells for tolerance induction in a murine EAE model, Xu et al. demonstrated complete protection from disease if mice received HSCs transduced with full length proteolipid protein (PLP) prior to immunization and could block disease progression if transduced HSCs were administered 12 days after immunization [63]. And importantly gene transfer to HSCs has been able to not only induce tolerance but also provide partial correction of disease phenotypes in hemophilia A [64–67], hemophilia B [68, 69], and Pompe disease [70, 71].

Table 15.1 Selection of disease models where gene delivery has been used to induce tolerance to the transgene product and the various strategies involved

Site of gene transfer	Strategy	Disease models
Liver	Protein replacement by liver gene transfer of FIX via AAV, Lentivirus, or retrovirus to neonate	Hemophilia A/B, Lysosomal storage disorders, antitrypsin deficiency, Crigler–Najjar type l,
	Plasmid or Ad-mediated expression of MBP to prevent induced autoimmune disease	Experimental autoimmune encephalitis (EAE, model of human multiple sclerosis)
	AAV-mediated gene transfer to both liver and brain to prevent antibody formation and improve outcome	Niemann–pick disease
Brain	Intracranial injections of AAV carrying therapeutic gene with does—dependent tolerance to vector along with escape from pre-existing antibodies	Parkinson disease, Canavan disease
Eye	Protein replacement by AAV-mediated gene transfer to subretinal epithelium to restore visual function	Leber congenital amaurosis
	Unsuccessful attempt to use antigen expression in the eye to prevent EAU	Experimental autoimmune uveoretinitis
HSCs	Lentiviral or retroviral gene transfer for expression of antigen to induce tolerance to expressed antigen and other antigen on transduced cells	Allograph/Xenograph transplantation, EAE prevention,
	Lentiviral or retroviral gene transfer for expression of therapeutic protein for both tolerance and phenotype correction	Hemophilia A/B, Pompe disease
B cells	Expression of antigen-lgGl fusion protein in B cell to mediate peripheral tolerance to antigen	Type l diabetes, EAE, Hemophilia A
T cells	Forced expression of FoxP3 to generate Treg that mediate tolerance to allograft	Type l diabetes, GVHV, contact hypersensitivity, male/female allograft, systemic autoimmunity
	Transfer of TCR specific for allograft recipient to Treg	Allograph

Modeled after LoDuca 2009 and Lowenstein 2009 [6, 88]

15.6 B cell Gene Transfer and Tolerance

B cells have been shown to be capable of antigen presentation and it has been repeatedly demonstrated that retroviral delivery of a fusion protein containing an antigen in frame with the IgG1 heavy chain leads to tolerance [72]. Extensive studies performed by David Scott's laboratory have identified several key requirements for B cell-mediated tolerance, including MHC class II presentation on B cells and the presence of Treg. B cell-mediated gene transfer has resulted in

successful tolerance induction in autoimmune models of multiple sclerosis and EAE [73, 74], type 1 diabetes in the NOD mouse model [73], and rheumatoid arthritis [75]. Additionally this B cell approach has been demonstrated to induce tolerance to coagulation FVIII in a murine Hemophilia A mouse model [76], in which both humans and mice with hemophilia A develop inhibitory antibodies following administration of FVIII protein [77]. The generation of IgG1 fusion proteins has the advantage of not requiring the identification of a specific epitope for each antigen and, as demonstrated with the FVIII protein, has the potential to prevent the generation of inhibitory antibodies to a variety of therapeutic proteins delivered either by gene transfer or as recombinant proteins.

15.7 T cell Gene Transfer and Tolerance

While gene transfer to HSCs and B cells has been extensively investigated for tolerance induction, there has been little effort placed on gene transfer to T cells until recently with the description of Treg cells ($CD4^+CD25^+$) and the identification of the FoxP3 gene as a master regulatory gene for the conversion of effector T cells ($CD4^+CD25^-$) into Treg cells [78–80]. Indeed, it was found that forced expression of FoxP3 can lead to the generation of cells that have similar suppressor function in vitro and in vivo as compared to naturally occurring Treg [81]. Thus, it has been shown that forced expression of FoxP3 has the ability to induce tolerance in a murine model of GvHD [82], inhibit contact hypersensitivity and autoimmunity in an autoimmune prone murine model [83], protect male skin grafts from rejection in syngeneic females [84], and prevent autoimmune diabetes in a NOD murine model [85]. In regards to transplantation, donor MHC molecules can be recognized on the surface of donor cells in two ways. Either directly, inducing an immune response and early transplantation rejection or indirectly after being internalized, processed and presented by recipient APCs, often leading to chronic graft rejection [86]. To address this indirect response, Tsang et al. introduced a specific TCR into Treg cells previously exposed to donor APCs, recognizing MHC class II presented alloantigen from donor MHC and demonstrated improved tolerance over Treg activated by the direct response [87].

In general it is possible to isolate effector T cells, expand these cells in vitro in the presence of the alloantigen, and, when sufficient numbers of cells are obtained, transduce these cells with a retro- or lentiviral vector expressing FoxP3 to generate Treg specific for the appropriate alloantigen. Experimental evidence suggests that monoclonal T cells are more effective at suppressing immune responses as opposed to polyclonal.

15.8 Conclusion

The applications of generating immune tolerance to the products of gene therapy range from enabling and/or enhancing long-term protein replacement such as in hemophilia and Pompe disease, to the potential treatment of immunological disorders like diabetes and arthritis (Table 15.1). By targeting specific anatomical locations and even specific cell types, it is possible to harness the unique role of these sites in the immune system as facilitators of tolerance. Each site (organ/cell type) will offer different advantages depending on the therapeutic goals. For example, if the goal is simply stable expression of a transgene, the brain, liver, eye, and hematopoietic cells may each be viable options. But if the primary goal is peripheral tolerance to an antigen, the liver and hematopoietic cells will be more desirable (Fig. 15.1). At the same time, the choice of target tissue may be limited by such factors as natural protein tropism, organ accessibility, vector tropism, and other disease-specific limitations, including tissue damage in degenerative diseases such as Duchenne muscular dystrophy.

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Chapter 16
Addressing the Challenge of Autoimmunity in the Treatment of Diabetes with Stem Cells

Karen English and Kathryn J. Wood

Abstract Type 1 diabetes mellitus is a complex autoimmune disease process encompassing a number of stages, the most significant of which is the loss of immunological tolerance and the initiation of immune dysfunction resulting in the selective destruction of pancreatic β cells. Although exogenous insulin therapy has proven efficacious, it does not address the underlying cause of the disease. A treatment strategy encompassing immunosuppressive and β cell replacement therapy that will promote immunological tolerance, without toxicity or the induction of lymphopenia is required for treatment of patients with hypoglycaemic unawareness. Importantly, this combination strategy must harness a therapy that provides a replacement source of insulin-producing β cells without toxic side effects associated with long-term immunosuppression and induces tolerance to the replacement β cells in order to prevent destruction by allo- and autoreactive T cells. Here, we discuss the current immunosuppressive therapies and potential sources of replacement β cells and review the pitfalls in current combined immunosuppression and islet transplant therapy. Finally, we examine possible combination strategies including stem cells that are likely to succeed in fulfiling the above criteria for the treatment of diabetes in the future.

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16.1 Introduction

Type I diabetes mellitus (T1DM) is an autoimmune disease in which autoreactive T cells target the pancreatic insulin-producing β cells. Destruction of β cells leads to insulin paucity, blood glucose dysregulation and subsequent hyperglycaemia which in turn result in a number of long-term micro and macro-vascular complications [1]. Diabetes is fast becoming a common childhood disease with the incidence set to double in children under the age of 5 by 2020 [2]. On a global scale, the World Health Organisation (WHO) estimate that 220 million people suffer from diabetes worldwide, a number which is set to increase twofold by 2030. The costs of treatment and the clinical management of the associated complications are substantial. The need for a preventative or regenerative therapy is perhaps at its most urgent since the discovery and development of insulin in the 1920s by Banting, Best, Collip and Macleod. Existing therapies include insulin therapy, cell-based therapy and solid organ transplantation as well as immunotherapy [3]. Although insulin therapy revolutionised the treatment of diabetes, there are limitations, particularly in the group of patients with hypoglycaemic unawareness. As insulin therapy does not address the cause of the disease, there is need for a therapy which has the capacity to address the autoimmune response, the replacement of insulin producing β cells and, if necessary, the problem of alloreactivity evoked by the replacement therapy.

A combination of genetic susceptibility [4] and environmental factors trigger changes in the immune system leading to immune dysregulation and subsequent autoimmunity accompanied by the development of islet specific autoantibodies and autoreactive T cells. The disease process starts with genetic susceptibility [4] (stage 1) followed by triggering events (stage 2). Immune dysregulation and environmental triggering (stage 3) are followed by the loss of β cell function detected by abnormal glucose tolerance test (stage 4). At diabetes onset (stage 5) almost 80 % of the beta cells are already lost. The final stage of this process (stage 6) is the total loss of β cells with patients dependent on insulin therapy for survival [5] (Fig. 16.1).

16.2 Current Therapeutic Strategies for Type 1 Diabetes Mellitus

A number of potential therapeutics have been investigated in pre-clinical models of diabetes with many demonstrating efficacy in prevention or reversal of T1DM, however, in the majority of cases, these results do not extrapolate to humans. Factors including variations in genetic predisposition, environmental triggers as well as inter-individual heterogeneity in disease pathogenesis all effect therapeutic outcomes [6–8]. Additionally, a major issue concerning the current replacement therapy for T1DM, namely islet or pancreas transplantation, is the problem of

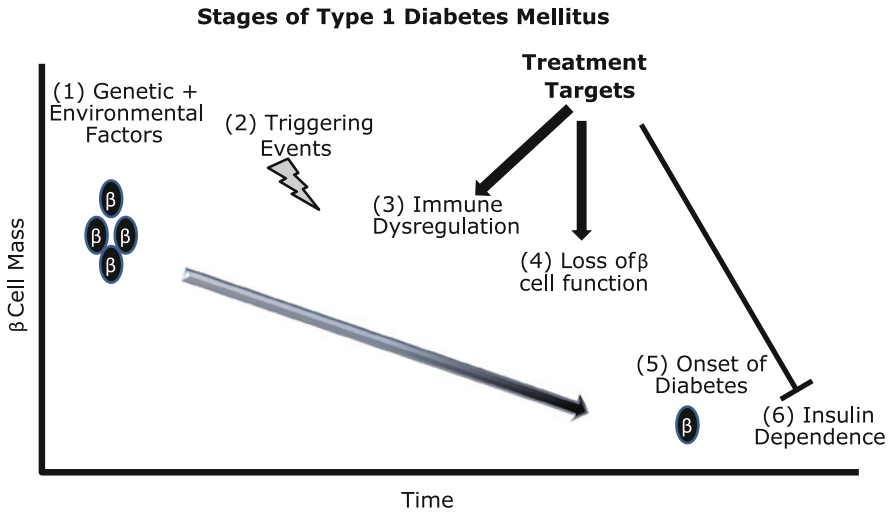

Fig. 16.1 Stages of Type 1 Diabetes. Diabetes is thought to be initiated by interactions between genetic susceptibility and environmental factors (*1*). Evidence suggests that triggering events such as enterovirus infection contribute to the pathogenesis of T1DM (*2*). Loss of immunological tolerance coincides with immune dysregulation (*3*) resulting in the activation of autoreactive T cells and subsequent destruction of β cells leading to loss of β cell function (*4*) and significantly decreased β cell mass signifying the onset of diabetes (*5*) which inevitably results in insulin dependence (*6*). Treatments are specifically targeted to stage (*3*) using immunosuppressive tolerance induction strategies and importantly stage (*4*/*5*) utilising β cell replacement therapy in an attempt to prevent insulin dependence

recurrent autoreactive T cells [9] which seem to be resistant to suppression by conventional immunosuppressive drugs. Here, we discuss the immunotherapies and potential sources of β cells for the treatment of diabetes patients focussing on the possibility of combination therapies of immunosuppressive agents that will effectively address autoimmunity and alloreactivity (Fig. 16.2).

16.3 Immunotherapy

16.3.1 Global Immunosuppression

Randomised trials initiated in the 1980s tested the effects of global immunosuppressive drugs in modulating autoimmune diabetes, including cyclosporine [10, 11], azathioprine alone [12], or in combination with prednisone [13], anti-thymocyte globulin and prednisone [14] and rituximab (humanised monoclonal antibody (mAb) specific for CD20) [15]. All of these strategies led to improved endogenous β cell function and a decrease in insulin requirements in patients with new onset diabetes. However, the beneficial effects were limited to the duration of the treatment and the

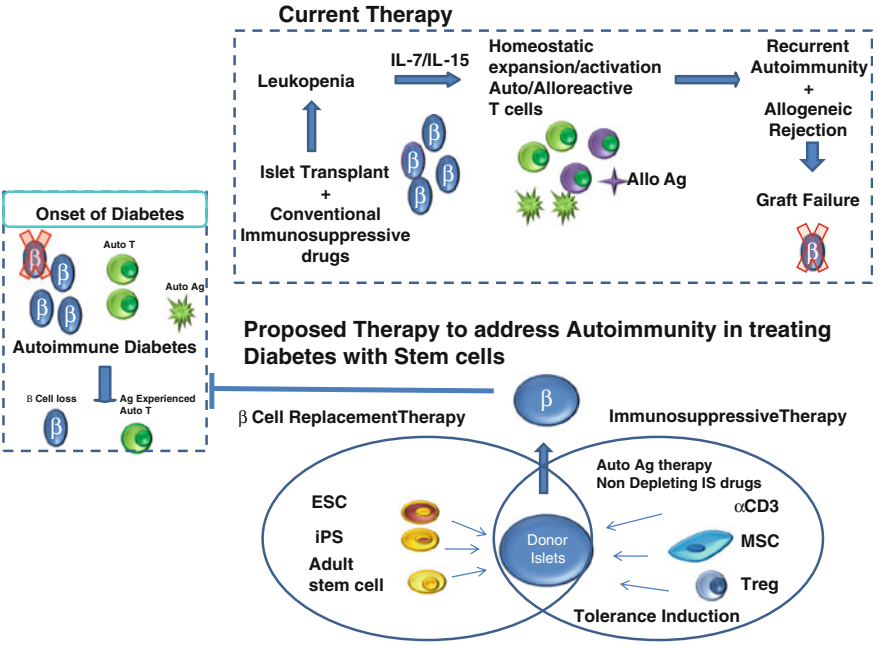

Fig. 16.2 Strategies of combination therapy for T1DM. The onset of diabetes is thought to be initiated by multiple factors involving genetic and environmental factors as well as triggering events which induce *β* cell death, initiating the process of autoimmunity—in which autoantigen and autoreactive T cells play a key role. Current therapy consisting of donor allogeneic islet transplant and conventional immunosuppressive drugs induce a state of leukopenia and promotes the homeostatic expansion and activation of pre-existing autoreactive T cells that target the transplanted islets for destruction and result in graft failure. Future therapy involving an ES cell, iPS cell or adult stem cell-derived *β* cell source in conjunction with a combination immunotherapy consisting of anti-CD3, MSCs, Treg, autoantigen therapy or non-depleting immunosuppressive drugs may provide a more successful outcome through avoidance of leukopenia and induction of tolerance

side effects associated with cyclosporine in particular [16] suggested that global immunosuppressive therapy alone was unsuitable for treatment of T1DM.

The standard immunosuppression used in whole pancreas transplantation can vary between different centres, however, a protocol involving thymoglobulin as induction, with tacrolimus, mycophenolate mofetil (MMF) and prednisone for maintenance therapy, results in 80–85 % of grafts maintaining function after 1 year [17]. In contrast, utilisation of the Edmonton immunosuppressive protocol (optimised over a number of years) in conjunction with transplantation of allogeneic human islets has proven successful in rendering 100 % of patients (n = 7) with T1DM insulin independent for at least 1 year and provides a prime example of the potential of immunosuppressive drugs (discussed in more detail below) [18].

16.3.2 Antigen-Specific Therapies

A number of diabetes related autoantigens including insulin, pro-insulin, insulin peptides, glutamic acid decarboxylase (GAD) and hsp60 and hsp peptides p277 have been utilised in tolerance induction strategies. The NOD mouse has provided a useful platform to test the efficacy of antigen-specific therapy in diabetes. Studies have demonstrated the ability of exogenous insulin, pro-insulin or insulin peptides delivered orally, subcutaneously, or intranasally to prevent or suppress diabetes [19–23]. Delivery of GAD [24–27] or hsp60 and hsp60 p277 peptide [28, 29] through similar routes, also revealed promising results in NOD mice. Extrapolation of these antigen-specific therapies from the NOD mouse into human clinical trials has, however, proven difficult. Trials performed in new onset diabetes patients using oral insulin or an altered peptide ligand of the 9–23 insulin B chain peptide (NBI-6,024) demonstrated no effect [30, 31].

16.3.3 T cell and Co-Stimulation Targeting Agents

A number of monoclonal antibodies targeting CD3, CD4, CD8 and $\alpha\beta$ T cells as well as major histocompatibility complex (MHC) class II, CD28 and CD154 have been examined for their capacity to prevent or reverse T1DM [32–38]. However, among these; anti-CD3 therapy has prevailed as the most promising so far.

16.3.4 Anti-CD3 Therapy

Anti-CD3 mAb therapy has successfully induced a permanent state of disease remission in a rodent model of T1DM. In these studies antigen-specific tolerance was induced with mice regaining full immune competence after a few weeks of treatment [33, 39]. Clinical trials tested the efficacy of two humanised Fc engineered monoclonal anti-CD3 antibodies called teplizumab (Hokt3γ1 (Ala-Ala)) [40, 41] and otelixizumab (ChAglyCD3) [42, 43]. A multi-centre, Phase II placebo-controlled trial consisting of 80 patients with new onset T1DM receiving a 6-day treatment of either otelixizumab or placebo was carried out. This trial demonstrated that the anti-CD3 antibody preserved β cell function. Patients receiving the antibody maintained significantly higher levels of endogenous insulin secretion than placebo controls at 6, 12, 18 and 48 months after treatment [42, 43]. However, after 24 months, the beneficial effect diminished over time with a decline in β cell function and increase in insulin dependence [43]. Similar results were observed in the teplizumab trail which entailed a 12 or 14 day mAb treatment with 2–4 days of incremental dose escalation to 10 days of a full dose of drug mAb. There were significant improvements in C-peptide responses accompanied by reduced haemoglobin A_{1c} (HbA_{1c}) and

insulin requirements in patients receiving the mAb, however, the effects waned after 2 years [40]. Furthermore a small open labelled phase IIb clinical trial demonstrated that a higher dose (40 % higher) of teplizumab resulted in increased adverse events without an improved efficacy of the drug [44].

It seems likely that autoreactivity was only transiently suppressed in these trials leading to the subsequent loss of additional β cells and the increased requirement for exogenous insulin with time after cessation of the therapy. A study carried out by Albamunits et al. clearly demonstrated that β cell replication is reduced after immune therapy using an anti-CD3 mAb resulting in progressive loss of β cell mass [45], similar to the functional decline observed in humans after treatment with anti-CD3 [43]. The mode of action of the anti-CD3 mAb remains unclear but alteration of lymphocyte migration or trafficking, rather than depletion, has been suggested [44].

Although this therapy has been the most promising to date, it is likely that a combination of immunomodulatory agents coupled with a β cell replacement strategy (or β cell regeneration strategy in patients with a small but sufficient β cell mass) will be more efficacious in addressing the multiple factors associated with current allogeneic islet transplantation and future β cell replacement providing a more successful outcome.

16.3.5 Regulatory T cell Therapy

Autoimmune diabetes manifests from the loss of immunological tolerance. T1DM patients as well as NOD mice, exhibit a decreased frequency of regulatory T cells (Treg) with alterations in function [46–49] and IL-2/IL-2R signalling pathway [50]. There is evidence to suggest that NOD mice have Treg that prevent diabetes development early on, but the functional capacity of the Treg is lost over time [51], allowing dysregulated autoimmune attack of insulin producing β cells [52, 53]. Similarly, Treg cells taken from peripheral blood of T1DM patients also exhibit defective suppressive functions in vitro [54].

Although it seems likely that endogenous naturally occurring Treg are functionally defective in diabetic mice, adaptive Treg present in NOD mice have been shown to suppress autoreactive T cells mediated by TGF-β [51] and therefore represent a possible target for tolerance induction in vivo. Treatment with anti-CD3ε antibody induced tolerance in NOD mice through the activation of adaptive Treg in a TGF-β-dependent manner [55], highlighting the possibility of driving a tolerance induction pathway through mAbs. TGF-β-producing Treg have also been implicated in tolerance induction strategies involving immunisation with plasmid DNA encoding GAD65, IL-4 and IL-10 [56] in a NOD mouse model.

The other option available involving Treg therapy, is the selective ex vivo expansion of polyclonal or antigen-specific Treg [57]. Tang et al. [58] have demonstrated that in vitro expanded antigen-specific Treg cells suppress autoimmune diabetes in the NOD mouse with greater potency than expanded

polyclonal Treg. Utilising a humanised mouse model, our lab has demonstrated the capacity of ex vivo expanded human Treg to prevent rejection of a life sustaining human islet allograft in streptozotocin induced diabetic mice (Wu, Wieckiewicz and Wood unpublished results). The data from animal models of diabetes supports the use of human Treg in the treatment of autoimmune diabetes, however, the major discrepancy is the ability to generate human antigen-specific Treg directed against autoantigen. Additionally, although Treg have proven efficacious in the NOD mouse, extrapolation to the clinic will not be without difficulty and it is likely that a combination therapy of Treg and a short-acting immunosuppressive drug like anti-CD3 may be efficacious in breaking autoimmunity or preventing islet/stem cell-derived β cell rejection.

16.3.6 Mesenchymal Stem Cells

Mesenchymal Stem Cells (MSCs) are adult stem cells found within the bone marrow (as well as many other tissues and organs) whose primary functions are to provide stromal support for hematopoietic stem cells and to act as a reservoir for the continuous turnover of mesenchymal lineages under regular conditions. Moreover, MSCs possess immunosuppressive characteristics which make these cells an attractive source for cellular immunotherapy both in transplantation and autoimmunity settings.

In the context of autoimmunity, MSCs have been shown to be efficacious in suppressing autoreactive T cell responses in mouse models of colitis [59], experimental autoimmune encephalomyelitis (EAE) [60] and collagen induced arthritis (CIA) [61] among others.

Furthermore, MSCs have proven therapeutically beneficial in a mouse model of T1DM [62, 63]. Administration of MSCs derived from BALB/c or non-obese resistant strain but not non-obese diabetic (NOD) mice, delayed diabetes onset in pre-diabetic recipients. This delay in disease onset was thought to be associated with the expression of PD-L1 by MSCs and the promotion of a Th2 type response in treated NOD mice [62]. Importantly, this study highlights the important fact that MSCs isolated from diabetic patients may not have the same immunosuppressive capacity as MSCs from healthy patients and, therefore, use of allogeneic MSCs may need to be considered in this case.

Considerable evidence supports the ability of MSCs to prevent allogeneic graft rejection [64]. A number of in vivo studies demonstrate the ability of MSCs to suppress alloreactive responses both in skin and heart allograft models [65, 66]. In a fully MHC-mismatched baboon skin transplant model, a single dose of donor MSCs administered intravenously resulted in prolonged skin graft survival (11.3 ± 0.3 days compared to 7.0 days in untreated controls). However, this effect was non-specific as third party grafts were also prolonged in a similar time scale [65]. Casiraghi and colleagues report that both donor and recipient derived MSCs have the capacity to prolong cardiac allograft survival in a semi-allogeneic but not

a fully MHC-mismatched cardiac allograft model. Protection of cardiac allografts was associated with the expansion of Treg cells and the abrogation of anti-donor Th1 activity. Significantly, this study highlighted the differences between donor and recipient derived MSCs and shows that pre-transplant intraportal administration of a single dose of MSCs proved more efficacious than the intravenous route [66]. Unfortunately, this is not something that we can test in human subjects and therefore we must learn what we can from the successful use of MSCs in steroid refractory graft versus host disease (GvHD) patients, which involves intravenous administration of MSCs of autologous or allogeneic origin [67].

The capacity of MSCs to prevent donor allogeneic islet graft rejection was investigated utilising a life-preserving mouse islet allograft model. This streptozotocin induced diabetes model allowed us to examine the effect of MSCs in suppressing an alloreactive effector T cell attack on transplanted allogeneic islets in an immunodeficient mouse. In the absence of MSCs, donor allogeneic islet grafts were rejected with a mean survival time of 30 days, however, in the presence of MSCs islet grafts were maintained long term with stable normoglycemia. The ability of MSCs to prevent rejection in this study is likely an attribute of co-localisation of MSCs with the islet graft associated with the provision of a local immunosuppressive milieu by MSCs in the locality of the islet graft. In this local microenvironment MSCs produce soluble factors, in particular MMP-2 and 9 which impair alloreactive T cell activation and expansion [68].

In addition to suppression of autoreactivity, MSCs also potently modulate alloantigen specific responses and thus provide a promising therapeutic strategy for the treatment of type 1 diabetes. MSCs are unique as a cellular therapy in that they have the capacity to address both the problem of autoimmunity and alloreactivity against a cell replacement therapy for diabetes patients. However, although MSC therapy has shown much promise in controlling both allo- and autoreactivity, there is little doubt that MSCS therapy alone will not be sufficient and will likely be more efficacious as a combination therapy; with a single dose of, for example, anti-CD3.

16.4 β Cell Replacement Therapy

16.4.1 Islet Transplantation

By far the most forward moving of the cell replacement therapies is allogeneic islet therapy, which involves the transplantation of deceased donor-derived islets percutaneously into the portal vein of the liver, combined with immunosuppressive drugs. Islet therapy has recently improved in efficacy through introduction of the Edmonton protocol [18]. The success of the Edmonton team involved an improved islet isolation technique combined with infusion of large numbers of freshly isolated islets. In addition, an altered immunosuppressive regimen avoiding steroids

(glucocorticoid therapy), and combining a reduced dose of tacrolimus (a calcineurin inhibitor which can inhibit insulin secretion) with the addition of sirolimus (mTOR inhibitor) [18] was implemented. Under this regimen, 68 % of patients receiving islet allografts maintained insulin independence at year 1. Unfortunately, insulin independence was not sustained long term with less than 10 % of patients remaining insulin independent at year 5. However, 80 % of patients had measurable levels of C-peptide indicating the continuous low level production of insulin [69], which allows improved glycemic control. The benefits associated with islet transplantation although small, supports the utility of this procedure in a select group of patients experiencing hypoglycaemic unawareness (patients who have lost significant β cell mass). Problems associated with this therapy are the inefficient isolation procedure and the requirement of at least two pancreatic donors, for which there are simply not enough donors available. This lack of islet tissue has lead to the proposed use of adult, embryonic or induced pluripotent stem (iPS) cells for replacement therapy.

16.4.2 Embryonic Stem Cells

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the blastocyst with the capacity for unlimited self-renewal in an undifferentiated state, and the ability to undergo induced differentiation into all three germ layers in vitro and in vivo. The ability of mouse ES cells to differentiate in vitro into functional insulin producing cells or islet like clusters which can recover and maintain normoglycemia in streptozotocin-induced diabetic mice has been demonstrated by a number of groups [70, 71]. Human ES cell differentiation into insulin producing cells has proven more difficult due to an inability of ES cells to generate definitive endoderm. However, Baetge's group have generated a protocol that promotes differentiation of human ES cell derived endodermal cells into cells expressing pancreatic markers [72]. Although this protocol did not lead to glucose responsive insulin producing cells in vitro, implantation of human ESC derived—endodermal derived—insulin positive cells in immunodeficient mice resulted in the production of insulin in response to glucose several months after transplantation [73]. This study was the first of its kind to demonstrate that insulin producing cells derived from human ES cells could maintain normoglycemia in a mouse model of streptozotocin-induced diabetes. However, this study also highlights the fact that further research is required in order to fully optimise the differentiation protocol and to ensure phenotypically and functionally stable insulin producing cells are induced.

Importantly, the issues that these studies do not address is that of autoimmune and alloreactive attack. In the case of alloreactivity against ES cell-derived tissue, it is likely that these cells/tissues will be recognised as foreign and subsequently rejected by the immune response. The immune response directed against fully differentiated insulin producing tissue derived from mouse ES cells was examined using a mouse model of streptozotocin-induced diabetes. In this study, functional

insulin producing cells were rejected in immunocompetent hosts and this response was mediated primarily by T cells [74] with evidence for involvement of both innate and adaptive components of the immune system [75–77] .

16.4.3 Induced Pluripotent Stem cells

Ground breaking research describing the factors required to reprogram adult cells back into iPS cells in 2006 [78] has paved the way for tissue engineering and therapeutic application. Initial problems concerning homogeneity and efficiency of the reprogramming process are now being addressed promptly by a number of groups. However, clinical application of iPS cell-derived tissue remains a long way off with safety concerns regarding the stability of the cells in vivo and the possibility of tumour formation. Recently Tateishi et al. reported that human iPS cells derived from skin fibroblasts could be differentiated into islet like clusters, which expressed insulin. Of four iPS cell lines, two differentiated into islet like cell clusters and one of these released low levels of C-peptide in response to glucose stimulation [79]. iPS cells have also been derived from adult cells from patients with disease and one group have reported the differentiation of skin biopsy-derived iPS cells into insulin producing cells. This study was more convincing with evidence that cells released human C-peptide (5 fold increase) in response to glucose stimulation, suggesting that insulin producing cells were functional [80].

16.4.4 Adult Stem Cells

Bone marrow-derived cells can differentiate into a number of lineages and have been demonstrated to play a role in regeneration and repair and therefore represent an attractive source for tissue engineering in T1DM. Transplantation of bone marrow-derived cells into streptozotocin-treated mice led to the instigation of endogenous pancreatic tissue regeneration resulting in insulin production and improved survival [81]. In contrast, a number of studies have suggested that bone marrow-derived cells differentiated into insulin positive cells, however, the stem cell community is still not convinced that this lineage switch is possible. Nonetheless, two studies in particular have demonstrated that bone marrow-derived islet-like clusters transplanted into streptozotocin-induced diabetic rodents have the capacity to control blood glucose and maintain normoglycemia which was reversed after removal of the graft [82, 83].

Umbilical cord blood has stimulated interest both as an immunomodulatory therapy and as a potential source of insulin-producing cells for use in T1DM. Islet-like clusters derived from human Wharton's jelly MSCs in umbilical cord matrix produced low levels of insulin and have some effect in controlling blood glucose and maintaining normoglycemia in vivo [84]. Overall, it seems likely that adult

stem cells may have the capacity to differentiate into insulin producing islet like cells, however, the process of trans-differentiation is as yet an unproven phenomenon and therefore it is unlikely that adult cells will provide the most useful/optimal source of replacement β cells.

16.5 Strategies to Address Autoimmunity in β Cell Replacement Therapy

The prevailing issues that need to be addressed in the treatment of T1DM with β cell replacement therapy are the presence of autoreactive T and B cells specific for islet cell antigens (autoantigens), as well as lymphocytes with the capacity to respond to mismatched MHC antigens (alloantigens) of the β cell donor. Additionally, depending on the source of β cells, there may be other antigens, as in the case of ES cell-derived β cells which could evoke immune activation; for example ES and iPS cells express surface antigens that disappear at later stages of development and which are not expressed by adult cells [85, 86]. Graft failure has been reported in a minority of transplant patients and has been attributed to chronic rejection. However, another reason for graft failure is the recurrence of autoimmunity which was initially described in patients receiving pancreas graft from HLA-identical siblings with no or reduced immunosuppression [87, 88]. A large study examining 100 grafts described autoimmune diabetes recurrence in $\sim$10 % of patients receiving donor grafts with immunosuppression [89]. More recently, the recurrence of T1DM after simultaneous pancreas-kidney transplantation, despite the use of immunosuppressive drugs, was reported to be associated with both autoantibodies and autoreactive T cells [9]. Furthermore, conventional immunosuppressive drugs currently used in allogeneic donor islet transplantation prevent rejection through the depletion of leukocytes. The immune system responds to this through production of common γ chain cytokines IL-7 and IL-15 which stimulate the expansion of any remaining lymphocytes in an effort to restore homeostasis [90–92]. This effect also results in the expansion of pre-existing autoreactive cells and can lead to destruction of transplanted islets [93, 94]. Indeed, the production of IL-7 was shown to promote the expansion of autoreactive T cells in response to a lymphopenic environment [93]. Moreover, examination of the serum from T1DM patients after islet transplant, revealed the presence of increased concentrations of IL-7 and IL-15 [95]. There is some evidence to suggest that certain immunosuppressive drugs will promote homeostatic proliferation while others may inhibit it. Two patients receiving MMF plus tacrolimus in place of sirolimus plus tacrolimus (due to sirolimus intolerance) displayed reduced proliferation with no change in IL-7 expression, indicative of the capacity of MMF, but not sirolimus, to block cell proliferation [95]. This phenomenon was also observed in 3 patients who received kidney-pancreas transplant with anti-thymocyte globulin induction therapy followed by MMF plus

cyclosporine A (CyA) or FK506 maintenance therapy [95], demonstrating that the immunosuppressive therapy utilised may impact on the outcome of islet or β cell replacement therapy [96].

A poor clinical outcome has also been associated with the presence of islet specific autoantibodies [97] indicating that assessment of autoantibodies present before transplant of replacement β cells will be important in the choice of (patient tailored) immunosuppressive therapy.

16.6 Conclusion

Although significant progress has been made in the field of immunotherapy to halt autoimmune T1DM, the development for a combination therapy encompassing both immunotherapy and β cell replacement therapy (currently donor islet transplantation but stem cell-derived β cell tissue in the future) has been somewhat elusive. Encouragingly, there are already a number of promising therapies available targeted at autoimmune diabetes, which, if combined, could provide a very successful therapy, the key to which is undoubtedly a tolerance induction strategy.

In summary, the issues which need to be addressed in the quest for this optimal therapy are (1) evaluation of the presence and scale of pre-existing autoreactive T cells in patients before transplant of replacement β cells; (2) examination of the effect of new immunosuppressive regimens on the activation and expansion of pre-existing autoreactive T cells after transplant and (3) development of new therapeutic agents that have the capacity to prevent rejection and regulate the pre-existing autoimmune response without inducing profound leukopenia.

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About the Editor

Dr Paul Fairchild began his research career in Oxford, where he studied for a doctorate in the Nuffield Department of Surgical Sciences, focussing on the immune response to organ allografts. After spending five years as a post-doctoral fellow investigating the etiology of autoimmune disease in the Department of Pathology, University of Cambridge, he returned to Oxford, where he is currently a University Lecturer in Pre-clinical Medicine within the Sir William Dunn School of Pathology and a Fellow of Trinity College. In 2008, Paul Fairchild founded the Oxford Stem Cell Institute (OSCI), for which he currently serves as Co-Director. As a highly interdisciplinary organization, the OSCI focuses on exploiting the properties of stem cells for the treatment of some of the most intractable chronic and degenerative diseases. It is within this context that he continues to apply his background in transplantation immunology, in order to investigate the nature of the immune response to tissues differentiated from pluripotent stem cells, and develop approaches to the induction and maintenance of immunological tolerance.

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