ADVANCES IN EXPERIMENTAL MEDICINE AND BIOLOGY

Volume 684

Memory T Cells

Edited by
Maurizio Zanetti
and Stephen P. Schoenberger

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DEDICATION

In memory of Nerita Zanetti (October 5, 2009).

PREFACE

Immunological memory has fascinated microbiologists and immunologists for decades as one of the new frontiers to conquer to better understand the response to pathogens, cancer and vaccination. Over the past decade, attention has turned to the intrinsic properties of the memory T cells themselves, as it has become clear that the eradication of both infected cells and tumors requires T cells. This book is an attempt to capture the wave of discoveries associated with these recent studies. Its chapters represent a wide collection of topics related to memory T cells by laboratories that have invested their skills and knowledge to understand the biology and the principles upon which memory T cells are generated, maintained and expanded upon re-encounter with antigen. Ultimately, these studies are all aimed at a better understanding of the function of memory T cells in protection against disease.

Little is still known about the transcriptional events regulating the emergence of memory T cells. Chapter 1 discusses the role of Schnurri-2, which plays a critical role in cell growth, signal transduction and lymphocyte development, in the generation of memory CD4 T cells. Nakayama and Kimura point out that Schnurri-2 mediates repression of NF-κB hence enabling cell survival during the critical time of memory cell generation. In Chapter 2, Rocha and colleagues focus on how transcriptional regulation in memory CD8 T-cell responses allows them to more efficiently respond to antigen stimulation by modifying their cell cycle machinery in order to divide faster, improving DNA repair and enacting other cell survival mechanisms.

The role of cytokines, novel costimulatory molecules and other signals coming from the microenvironment in the generation and maintenance of memory T cells is discussed in Chapters 3-5. In Chapter 3, Sprent and colleagues discuss the importance of IL-2 signaling during the contraction phase in memory CD8 T-cell differentiation. At the time where most antigen-specific CD8 T cells disappear by apoptosis, IL-2 signals rescue CD8 T cells from cell death and provide a durable increase in cell counts. This work shows that IL-2 signals during different phases of an immune response are key in optimizing CD8 T-cell functions, affecting both primary and secondary responses. Chapter 4 sees Butler and Harty discussing the role of inflammation in the generation and maintenance of memory T cells showing that these two phases are influenced by a multitude of factors, including inflammatory cytokines that can

act on T cells during their differentiation, actually shaping the quantity and quality of memory T cells, and the rate at which functional memory populations develop. Chapter 5 points to a critical role of OX40 (CD134) in T-cell memory generation. Weinberg argues that enhancing OX40 signaling during antigen priming using OX40 agonists increases memory T-cell development. He also discusses the therapeutic potential of OX40 agonists and antagonists in human clinical trials.

Chapters 6-9 discuss the generation and maintenance of memory CD8 T cells during acute or chronic viral infection, in the context of the efficiency of recall responses and protection. In Chapter 6, Marzo, Sowell, and Scott seek to establish a correlation between precursor frequency and the differentiation of memory T cells following acute viral infection. In Chapter 7, Ahmed and colleagues discuss the parameters that influence memory CD8 T-cell generation and maintenance in the context of acute vs. chronic viral infection. They also discuss new surprising findings linking mTOR function with the generation of memory T cells. In the same vein Walker and Sifka in Chapter 8 examine the longevity of memory CD8 T cells after acute viral infection by a variety of methods. Their goal is to identify the optimal combination of functional characteristics required for protective immunity against the infectious disease. In Chapter 9, Zanetti and colleagues review the available information to formulate a set of principles to guide the generation of more effective vaccines that elicit protective memory T cells. The working principles for the generation of protective memory T cells by vaccination are discussed both in the context of the immunologically-inexperienced and immunologically-experienced individual.

In Chapter 10, Vaccari and Franchini present and discuss data on the induction of memory T-cell responses in non-human primates, and argue that these experimental models yield information that more closely matches the events that take place in humans with respect to induction and maintenance of memory T cells. Colpitts and Scott in Chapter 11 examine a few experimental models used to elucidate the nature of the memory T cells that are generated during parasitic infections, and suggest that long-term immunity induced by vaccination is a realistic goal to control parasitic infections.

Chapters 12-15 review what is currently known about the role of memory T cells in cancer and provide hints about monitoring memory T-cells responses after vaccination. In Chapter 12, Alderson and Murphy discuss how memory T-cell responses generated by vaccination are impacted upon by concomitant immune stimulation by adjuvants of novel formulation or cytokines. The authors also discuss whether initial tumor regression and generation of sustained anti-tumor immunity have different immunological signaling requirements. Chapter 13 summarizes a survey in colorectal cancer patients correlating the presence of memory T cells infiltrating tumors in humans and clinical outcome. Camus and Galon provide evidence that a strong and coordinated in situ T-cell response, with accumulation of memory T cells within primary tumors, dramatically reduces the risks of relapse. This highly significant correlation between the quality of the in situ immune response, tumor dissemination, and clinical outcome suggests that attempts to vaccinate cancer patients may ultimately

succeed if the effect documented in the natural evolution of colorectal cancer can be generalized to other forms of cancer. Chapter 14 presents a state-of-the-art review by Marincola and colleagues of current methods to monitor T-cell immune responses to vaccination in cancer patients. Ex vivo assays to evaluate tumor-specific T-cell responses at the immunological and transcriptional level have demonstrated tumor recognition and T-cell activation, implying that tumor-specific T-cell induction indeed occurs in vivo. However, at the present time these assays alone are not adequate to predict tumor regression.

Finally, in Chapter 15, Henson and Akbar discuss a crucial issue in the biology of memory T cells: memory T-cell homeostasis and senescence during aging. The authors argue that because life expectancy has dramatically increased and continues to increase, memory T cells will also have to span over longer times. Our current understanding is that T-cell memory wanes during aging as evidenced by the susceptibility of old individuals to infection by organisms to which they were previously immune. They show that this loss of memory T cells originates from antigen-driven differentiation and exhaustion, and telomere erosion.

The field of study of memory T cells is relatively young and the unknowns are still numerous, but the intellectual and medical rewards promised by a better understanding of the biology of memory T-cell generation, differentiation and maintenance, are formidable not only to understand the immunodynamics of natural adaptive T-cell responses but also for the induction of protective T-cell responses by vaccination.

The compendium of topics assembled in this book and the ideas discussed in it represent our initial effort to lay the foundation for a comprehensive view of the field and its future potential. The scientific facts and premises presented here constitute, in our view, the seeds from which new work will originate hopefully increasing our knowledge and ultimately having an impact on our fight against many human diseases.

We thank Ronald G. Landes for having encouraged us to undertake this project.

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CONTENTS

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Chapter 1

Memory Th1/Th2 Cell Generation Controlled by Schnurri-2

Toshinori Nakayama* and Motoko Y. Kimura

Abstract

Schnurri (Shn) is a large zinc-finger containing protein, which plays a critical role in cell growth, signal transduction and lymphocyte development. There are three orthologues (Shn-1, Shn-2 and Shn-3) in vertebrates. In *Shn-2*-deficient mice, the activation of NF-κB in CD4 T cells is upregulated and their ability to differentiate into Th2 cells is enhanced in part through the increased expression of GATA3. Shn-2 is found to compete with p50 NF-κB for binding to a consensus NF-κB motif and inhibit the NF-κB-driven promoter activity. In addition, Th2-driven allergic airway inflammation was enhanced in *Shn-2*-deficient mice. Therefore, Shn-2 appears to negatively control the differentiation of Th2 cells and Th2 responses through the repression of NF-κB function. Memory Th1/Th2 cells are not properly generated from *Shn-2*-deficient effector Th1/Th2 cells. The expression levels of CD69 and the number of apoptotic cells are selectively increased in *Shn-2*-deficient Th1/Th2 cells when they are transferred into syngeneic host animals, in which memory Th1/Th2 cells are generated within a month. In addition, an increased susceptibility to apoptotic cell death is also observed in vitro accompanied with the increased expression of FasL, one of the NF-κB-dependent genes. Th2 effector cells overexpressing the p65 subunit of NF-κB demonstrate a decreased cell survival particularly in the lymph node. These results indicate that Shn-2-mediated repression of NF-κB is required for cell survival and the successful generation of memory Th1/Th2 cells. This may point to the possibility that after antigen clearance the recovery of the quiescent state in effector Th cells is required for the generation of memory Th cells. A repressor molecule Shn-2 plays an important role in this process.

Introduction

The effector helper T (Th) cells can be categorized into at least three subsets in function, Th1, Th2 and Th17 cells. Th1 cells produce IFNγ and direct cell-mediated immunity. Th2 cells produce IL-4, IL-5 and IL-13 and play critical roles in allergic reactions. Th17 cells are involved in certain autoimmune diseases. The differentiation and the function of these Th cell subsets are governed by several critical transcription factors. Among them, GATA3 appears to be a master transcription factor for Th2 cell differentiation,[1,2] T-bet for Th1[3] and RORγt for Th17.[4]

The generation of memory T cells is crucial for adaptive immunity and protection from infectious disease upon subsequent exposure to pathogens. Figure 1 illustrates the cellular processes that are required for the generation of functional memory Th1/Th2 cells.[5] Upon antigen recognition, naïve CD4 T cells undergo clonal expansion and differentiate into effector Th1/Th2 cells. After antigen clearance, the majority of these expanded effector Th1/Th2 cells undergo apoptotic cell death at the contraction phase.[6] Some of the effector cells survive for a long time in vivo as memory

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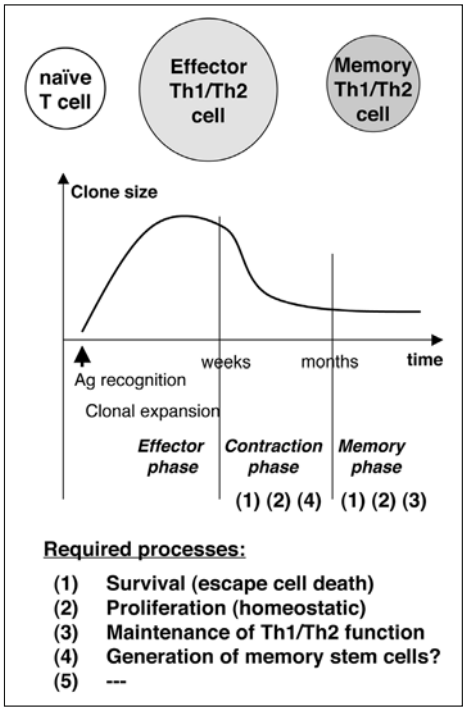

Figure 1. Cellular processes required for the generation of functional memory Th1/Th2 cells. The details are described in the text. Reproduced from: Nakayama T, Yamashita M. Curr Opin Immunol 2008; 20(3):265-271; with permission from Elsevier.[5]

type Th1/Th2 cells. In developing memory Th cells, several processes, such as (1) cell survival/escape from cell death, (2) proliferation/homeostatic proliferation and (3) the maintenance of Th1/Th2 cell function are required for the successful generation of functional memory Th1/Th2 cells (Fig. 1).

This chapter summarizes the recent findings on the role of an interesting zinc finger repressor, Schnurri-2 (Shn-2) in the generation and maintenance of memory Th1/Th2 cells. Shn-2 appears to downregulate the NF-κB target genes to maintain a quiescent state and support cell survival of developing memory Th cells at the contraction phase to facilitate the successful generation of memory Th1/Th2 cells.

Schnurri Family Genes

Schnurri (Shn) is a large zinc finger-containing protein; the molecular mass of Shn is ~270 kDa (Fig. 2). Shn was originally reported to be a nuclear target in the *Drosophila* decapentaplegic (Dpp) signaling pathway and interacting with Mad-Medea.[7-9] In vertebrates, the *Drosophila* Dpp signaling pathway may equate to the bone morphogenetic protein/TGF-β/activin signaling pathways that play various roles in developmental processes.[10] Vertebrates have at least three orthologues of Shn: Shn-1 (also known as HIV-EP1, MBP-1, PRDII-BF1 and αA-CRYBP1), Shn-2 (also known as HIV-EP2, MBP-2, AGIE-BP1 and MIBP1) and Shn-3 (also known as HIV-EP3, KRC and ZAS3). Although the analysis of Shn-1 in the immune system has not been reported, those for Shn-2 and Shn-3 substantially investigated.

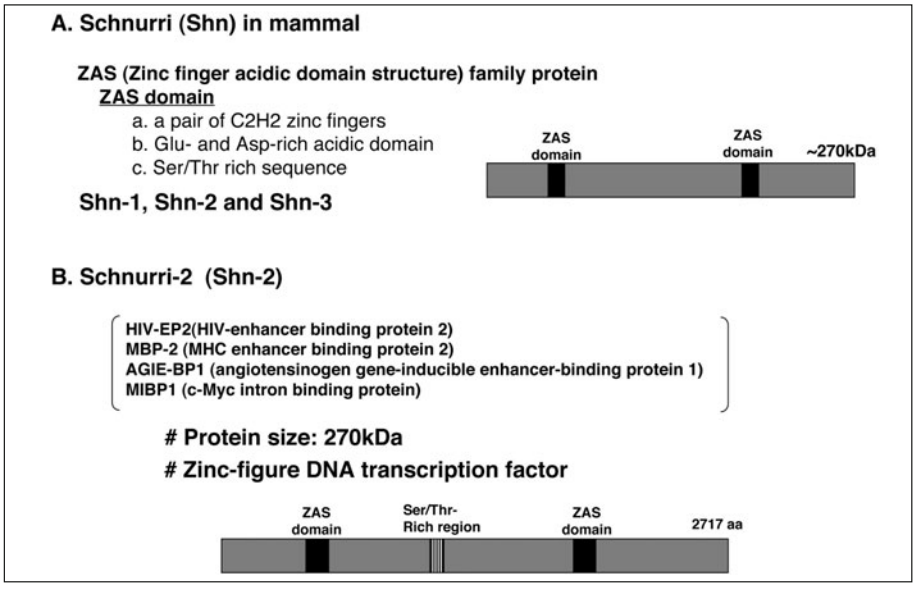

Figure 2. Schematic feature of Schnurri.

Shn-3

Shn-3 is the most precisely analyzed molecule among the Shn proteins. It was originally identified as a DNA-binding protein of the heptameric recombination signal sequence required for VDJ recombination of immunoglobulin genes.[11] Shn-3 can bind to the NF-κB motif directly and inhibit NF-κB activation.[12] Shn-3 interacts with an adopter protein TRAF2 and controls TNF receptor-driven responses.[13] Oukka et al demonstrate that the overexpression of Shn-3 inhibited while dominant-negative Shn-3 enhanced NF-κB-dependent transactivation and JNK phosphorylation after TNFα stimulation and regulates the apoptotic cell death and the expression of cytokine genes. Shn-3 also interacts with c-Jun to augment AP-1-dependent IL-2 gene transcription in T cells. The overexpression of Shn-3 in transformed and primary T cells leads to increased IL-2 production, whereas Shn-3 deficient T cells produce decreased IL-2.[14] Moreover, the expression of Shn-3 in the regulation of the adult bone mass has previously been reported.[15] Shn-3 deficient mice have markedly increased bone mass by promoting Runx2 degradation through the recruitment of E3 ubiquitin ligase WWP1 to Runx2. The survival of Shn-3-deficient $CD4^+CD8^+$ double positive thymocytes was reported to be decreased,[16] while there was no effect for positive selection in the thymus.[14]

Shn-2

The mRNA expression of *Shn-2* was detected mostly in the brain, heart and immune cells.[17-19] *Shn-2*-deficient mice revealed several important physiological roles of Shn-2 in the immune system. First, Shn-2 is required for positive selection but not negative selection of T cells in the thymus.[20] This defect in positive selection is caused by Shn-2 deficiency in thymocytes but not caused by the deficiency in the thymic stroma cells. In mature T cells, Shn-2 regulates Th2 cell differentiation by controling GATA3 expression through the regulation of NF-κB activation.[21] Shn-2 also regulates memory Th cell generation (described below in detail). Shn-2 is also required for bone development is also reported.[22] Interestingly, however, Shn-2 deficient mice have the opposite phenotype in comparison to that of Shn-3 deficient mice. Shn-2 deficient mice have reduced bone remodeling and osteopenia by suppressing NFATc1 and c-fos expression. Shn-2 is involved in the BMP signaling in mammals. Shn-2 interacts with Smad1/4 and C/EBPα upon BMP-2 stimulation and

induces the expression of PPARγ2, a key transcription factor for adipocyte differentiation. Shn-2 deficient mice show a reduced amount of the white adipocyte tissue.[23]

Together, these results indicate that each Shn family gene shares some roles but also the work in different ways and are involved in many different physiological processes.

Role of Shn-2 in Naïve CD4 T and Effector Th2 Cells

Shn-2 deficient mice were mated with OVA-specific TCR Tg (DO11.10 Tg) mice and naïve CD4 T cells were subjected to in vitro stimulation of Th1/Th2 cell differentiation with OVA peptide and APC.[21] As shown in Figure 3A, Th2 cell differentiation was significantly enhanced in Shn-2

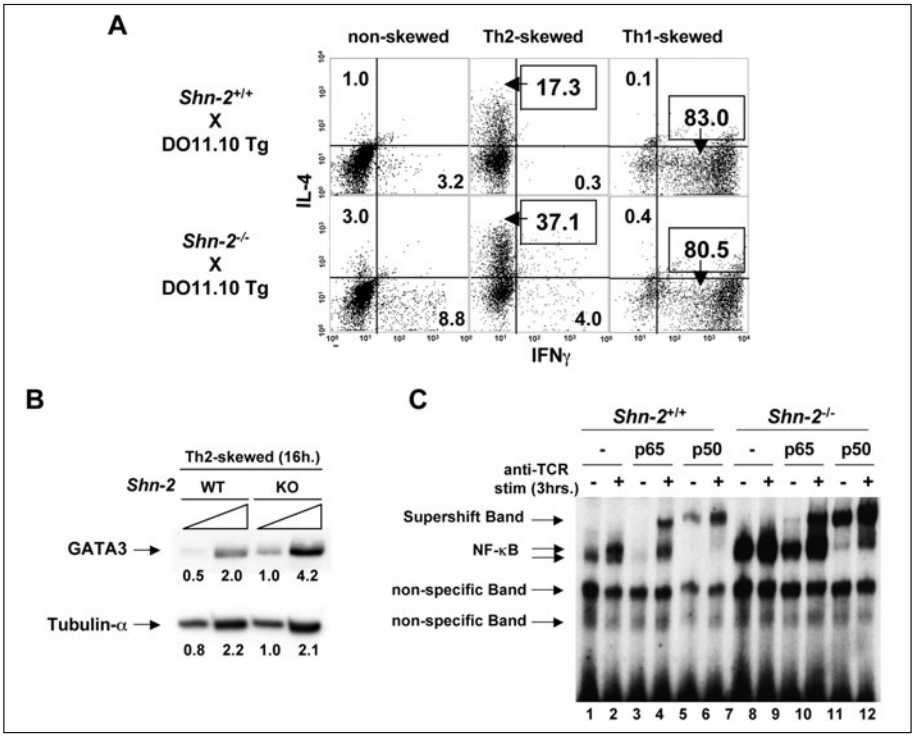

Figure 3. Enhanced Th2 cell differentiation accompanied with increased NF-κB activity and GATA3 expression in Shn-2-deficient CD4 T cells. A) Naïve ($CD44^{low}$) CD4 T cells from Shn-2-deficient (*Shn-2–/–*)x DO11.10 Tg mice were purified by cell sorting and stimulated with antigenic OVA peptide (Loh15: 0.1 μM) and irradiated BALB/c APCs for 5 days. Th2-skewed (IL-4 with anti-IL-12 mAb and anti-IFNγ mAb), Th1-skewed (IL-12 with anti-IL-4 mAb) and nonskewed (IL-2 with anti-IL-4 mAb, anti-IL-12 mAb and anti-IFNγ mAb) conditions were used. Intracellular staining was performed with FITC-conjugated anti-IFN-γ mAb and PE-conjugated anti-IL-4 mAb. Th2 cell differentiation is significantly increased in Shn-2-deficient CD4 T cells. B) Splenic CD4 T cells were cultured under Th2-skewed conditions for 16 hours. The cells were harvested and performed immunoblotting using anti-GATA3 or anti-Tubulin-α antibodies. Arbitrary densitometric units are shown under each band. GATA3 expression is increased in Shn-2-deficient cells. C) Splenic CD4 T cells were incubated with medium alone overnight and then stimulated with immobilized anti-TCRαβ mAb for 3 hours. Nuclear extracts of the cultured cells were prepared and subjected to EMSAs with NF-κB probes. The supershift assays were performed with antibodies specific for NF-κB p50 and p65 subunit detection. Hyper-activation of NF-κB was observed in Shn-2-deficient CD4 T cells. Reproduced from: Kimura et al, 2005. Originally published in *The Journal of Experimental Medicine*. doi:101084/jem.20040733.[21]

deficient T-cell cultures, leaving Th1 cell differentiation unaffected. Based on this observation, the in vivo consequence of the enhanced Th2 cell differentiation was assessed using Th2-driven allergic inflammation models. Shn-2 deficient mice showed enhanced Th2-dependent airway inflammation and airway hyperresponsiveness.[24]

The expression of GATA3, a master transcription factor for the differentiation of Th2 cells is induced in developing Th2 cells through TCR and IL-4 signaling.[25,26] In Shn-2 deficient T cells, the expression of GATA3 at the early time point (e.g., 16 hours after stimulation) after TCR and IL-4 stimulation is up-regulated (Fig. 3B). The induction of GATA3 expression is induced through the activation of the IL-4/Stat6 mediated signal.[27] Stat6 deficient T cells fail to up-regulate GATA3 expression and subsequently Th2 cell differentiation. However, no defects in Stat6 activation after IL-4 stimulation were detected in Shn-2 deficient T cells (data not shown). In addition, no obvious defect in intracellular Ca^{2+} influx or Erk1/Erk2 phosphorylation were detected upon TCR stimulation (data not shown), which are reported to be important for the efficient generation of Th2 cells.[28,29]

Another candidate molecule, which is involved in GATA3 expression, is NF-κB. An important role of NF-κB activation in GATA3 expression was originally reported in the allergic asthma model.[30] NF-κB p50 deficient T cells do not up-regulate GATA3 in in vivo OVA-induced airway inflammation model. In addition, protein kinase C (PKC) θ regulates NF-κB activation and GATA3 expression. In PKCθ-deficient CD4 T cells, the expression of GATA3 is severely impaired and Th2 cytokine production decreases.[31] Therefore, the expression of GATA3 appears to be controlled by NF-κB activation in peripheral CD4 T cells. As shown in Figure 3C, enhanced activation of NF-κB in both resting and activated T cells (resting cells: lane 1 vs lane 8, activated cells: lane 2 vs lane 9) is observed in Shn-2 deficient T cells. In addition, the protein expression of NF-κB (p50 and p65) in both cytoplasmic and nuclear fractions is equivalent between wild-type and Shn-2 deficient cells. Shn-2 directly binds to the NF-κB motif and inhibits the activation of NF-κB. Therefore, the enhanced NF-κB activation in Shn-2 deficient cells appear to be due to the increased binding of NF-κB but not due the increased protein expression of NF-κB (p50 and p65).[21]

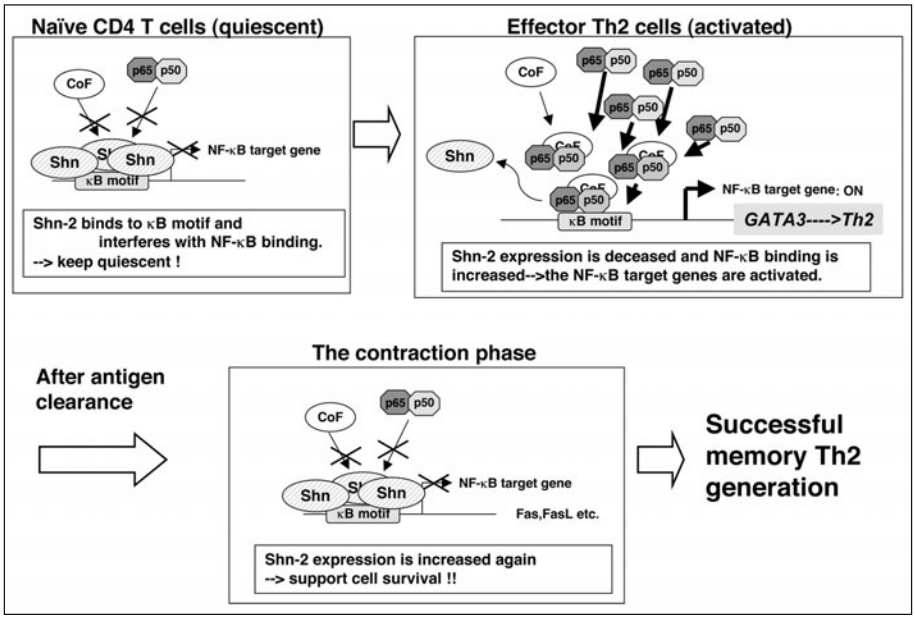

Figure 4. Shn-2 and NF-κB activation in naïve, effector and developing memory Th1/Th2 cells. The details are described in the text.

The molecular events operating in naïve CD4 T cells (quiescent cells) and effector Th2 cells (activated cells) can be illustrated based on these experimental results (Fig. 4, upper two panels). In naïve CD4 T cells, the expression of Shn-2 is very high (see Fig. 5) and Shn-2 constitutively binds to the NF-κB motif resulting in the expression of the suppressed NF-κB-dependent genes. Through this, naïve CD4 T cells are able to remain quiescent. However, once CD4 T cells receive antigenic stimulation through TCR, Shn-2 expression is decreased (see Fig. 5) and NF-κB signaling is activated and increased binding of p65/p50/cofactor is induced. As a result, the transactivation of the NF-κB target genes including GATA3 (Fig. 4, upper right) is induced in developing effector Th2 cells.

Role of Shn-2 in The Generation of Memory Th1/Th2 Cells

The unique expression of Shn-2 in naïve, effector and memory Th2 cells is schematically illustrated in Figure 5A. The expression levels of Shn-2 are high in naïve CD4 T cells and decreased after TCR stimulation and increased again in memory Th1/Th2 cells, particularly in memory Th2 cells (Fig. 5B). The expression of Shn-2 increased quickly even 3 days after cell transfer (Fig. 5C).[21]

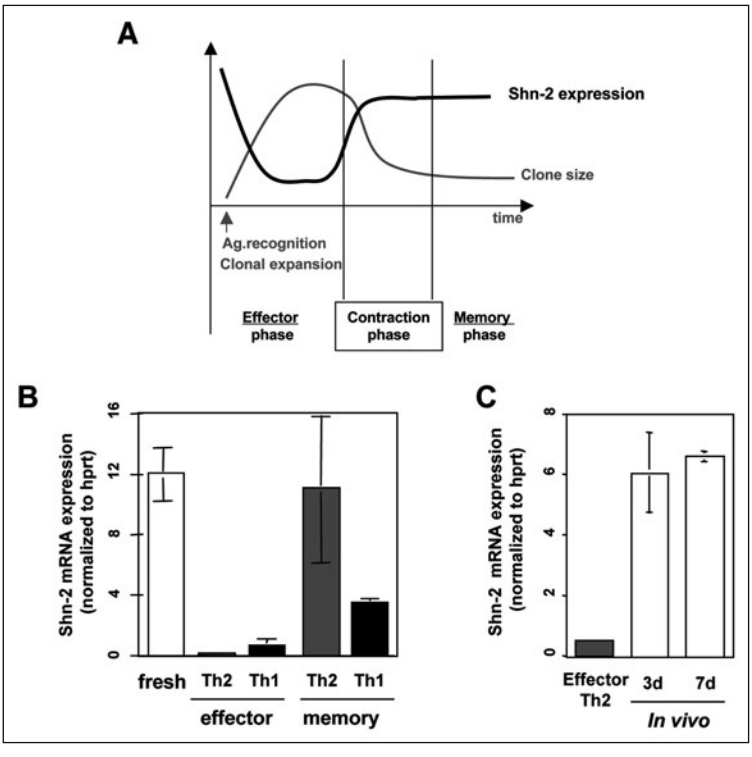

Figure 5. Shn-2 expression in the process of the generation of memory Th1/Th2 cells. A) Schematic representation of Shn-2 expression and clone size during the immune responses. B) mRNA expression of Shn-2 in fresh CD4 T cells from DO11.10 Tg mice, in vitro generated DO11.10 Tg effector Th1/Th2 cells and memory Th1/Th2 cells generated in BALB/c *nu/nu* mice was determined by a real-time PCR analysis. Shn-2 expression is high in fresh CD4 T cells, decreased in effector T cells and re-expressed in memory T cells. C) In vitro generated DO11.10 Tg effector Th2 cells were transferred into BALB/c *nu/nu* mice and 3 or 7 days later $CD4^+$ $KJ1\text{-}26^+$ transferred cells were purified and their mRNA expression of Shn-2 was assessed. Note that Shn-2 expression is recovered quickly after cell transfer. B,C) Reproduced with permission from: Schnurri-2 controls memory Th1 and Th2 cell numbers in vivo. J Immunol 178:4926-4936.

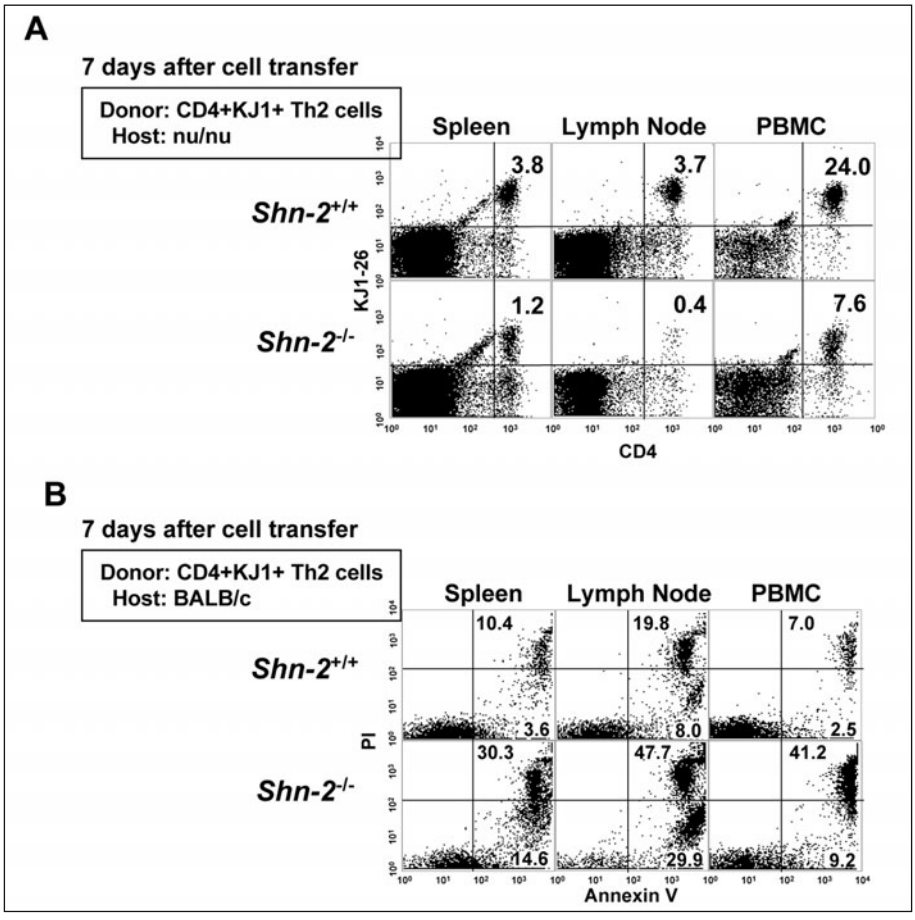

Figure 6. Decreased generation of Shn-2-deficient Th2 cells in the second lymphoid tissues. In vitro differentiated effector Th2 cells from DO11.10 Tg Shn-2-deficient mice were transferred into BALB/c *nu/nu* mice and the mice were analyzed 7 days after cell transfer. A) Representative profiles of CD4/KJ1-26 in the indicated organ are shown. Note that the percentages of Shn-2-deficient transferred Th2 cells were decreased. B) Annexin V/PI staining profiles were determined in electronically gated $CD4^+KJ1\text{-}26^+$ donor cells. Shn-2-deficient Th2 cells show an increased number of apoptotic cells. Reproduced with permission from: Schnurri-2 controls memory Th1 and Th2 cell numbers in vivo. J Immunol 178:4926-4936.

In order to address whether Shn-2 plays a crucial role in the generation of memory Th cells, an in vivo memory T-cell generation assay was performed using adoptive transfer of effector Th1/Th2 cells into syngeneic mice.[32] Interestingly, the numbers of Shn-2 deficient Th2 cells significantly decreased in the spleen, lymph nodes and PBMC 7 days after cell transfer (Fig. 6A).[21] No difference was detected in the liver and lung at this time point (data not shown). It is possible that Shn-2 deficient Th2 cells proliferate less effectively in vivo. To test this possibility, BrdU was administered three days after cell transfer and the incorporation of BrdU was analyzed. There is no decrease but rather slightly increased BrdU incorporation in Shn-2-deficient cells (data not shown). Consequently, the susceptibility to cell death of Shn-2 deficient T cells was examined and apparently increased Annexin V^+ cells in the spleen, lymph nodes and PBMC were observed (Fig. 6B). In addition, there was a high CD69 and FasL expression on Shn-2 deficient Th2 cells 7 days after cell transfer (data not shown). Similar

results were observed in an in vitro culture system. After overnight culture of effector Th2 cells in medium alone, anti-TCR stimulation, or in the presence of IL-7 for 3 days, significantly increased Annexin V^+ cells were detected in Shn-2 deficient Th2 cells. Again, increased levels of CD69, FasL and a slightly increase in Bim expression were detected in Shn-2-deficient Th2 cells. However no decrease was detected in Bcl-x, Bcl2 or Mcl1. The same effect was observed in Th1 cells. These results indicate that Shn-2 deficient T cells show a sustained activated phenotype and are more susceptible to die in vivo and in vitro.

NF-κB Overexpression in Effector Th Cells Results in the Decreased Generation of Memory Th Cells

Shn-2-deficient T cells show enhanced NF-κB activation (Fig. 3C) and decreased memory cell generation because of the increased apoptotic cell death (Fig. 6). Therefore, the decreased memory cell generation could be due to the enhanced NF-κB activation in Shn-2-deficient T cells. Th2 cells overexpressing p65 showed up-regulation of CD69 and FasL even in resting culture with medium. Transfer of the p65 overexpressing Th2 cells resulted in a selective decrease in the cell number in lymph nodes. Thus, Shn-2-mediated repression of NF-κB activation appears to be required for the generation of memory Th cells, particularly those in lymph nodes.

The molecular events operating in the Th cells at the contraction phase are illustrated in Figure 4, lower panel. After antigen clearance, and NF-κB activation decreased the expression of Shn-2 increased. As a result, the transactivation of the NF-κB target genes such as Fas and FasL is decreased and supports Th cell survival to generate memory Th2 cells successfully.

Even at the memory phase (e.g., one or two months after cell transfer), the Shn-2-mediated repression of the NF-κB activation in Th2 cells appears to be required for the maintenance of the proper number of memory Th1/Th2 cells (unpublished observation).

Interesting Questions Raised by the Study on Shn-2

Several interesting observations were noted during the analysis of Shn-2 in memory Th generation. First, the most prominent effect (decreased memory Th cell generation) of Shn-2 deficiency was detected in the lymph node. Shn-2 deficient T cells express decreased levels of CD62L. CD62L is known to be a homing receptor for the lymph nodes and the expression of CD62L is negatively controlled by the activation of NF-κB. Therefore, it was possible that the decreased Shn-2 deficient memory Th cells in the lymph nodes is due to the decreased homing of Th cells due to the low expression of CD62L. However, this appears not to be the case because the forced expression of CD62L in Shn-2-deficient effector Th cells did not rescue the number of Shn-2-deficient donor cells in the lymph nodes. Another possibility is that T-cell interaction with antigen-loading antigen presenting cells occurs most efficiently in the lymph node resulting in the increased activation induced cell death (AICD).

Second, the defect in the generation of memory cells was more prominent on $CD62L^{high}$ central memory phenotype cells in comparison to $CD62L^{low}$ effector memory like cells.[21] It is likely that the maintenance of $CD62L^{high}$ central memory phenotype cells is more dependent on Shn-2-mediated repression of NF-κB and resulting induction of the quiescence state. However, there is a more interesting possibility at this time. A nonlinear model has been proposed for the differentiation fate of central and effector memory cells[33] (see the chapter by Franchini et al). In this model, it is proposed that the levels of a certain transcription factor determine the differentiation fate of central or effector memory T cells. Therefore, it is likely that Shn-2 is the first example of a transcription factor, which determines the differentiation fate of central or effector memory T cells. Shn-2 may direct the differentiation of central memory Th cells.

Conclusion

A series of studies on Shn-2 shed light on several interesting aspects in the development of the memory Th cell system. First, Shn-2 is the first example of a transcription factor that controls cell survival to support the generation of memory Th cells. Second, transcription factors that are

induced in activated T cells are required to be suppressed for the proper formation of memory Th cells. Shn-2-mediated downregulation of NF-κB target genes would be a good example of this possibility. Third, the maintenance of the quiescent state in T cells (in resting memory Th cells as well as in naïve CD4 T cells) is an active process mediated by repressor molecules such as Shn-2.

Acknowledgements

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Chapter 2

Transcriptional Regulation during CD8 T-Cell Immune Responses

Ivana Munitic, César Evaristo, Hsueh Cheng Sung and Benedita Rocha*

Abstract

Naïve CD8 T cells differentiate in response to antigen stimulation. They acquire the capacity to express multiple effector molecules and mediate effector functions that contribute to infection control. Once antigen loads are reduced they revert progressively to a less activated status and eventually reach a steady-state referred to as "memory" that is very different from that of naïve cells. Indeed, these "memory" cells are "ready-to-go" populations that acquired the capacity to respond more efficiently to antigen stimulation. They modify their cell cycle machinery in order to divide faster; they likely improve DNA repair and other cell survival mechanisms in order to survive during division and thus to generate much larger clones of effector cells; finally, they also mediate effector functions much faster.[1] These modifications are the consequence of changes in the expression of multiple genes i.e., on the utilization of a new transcription program.

The Regulation of Gene Expression: General Rules

Multi-cellular organisms possess a single genetic code but multiple cell types, each of them defined by the expression of a particular set of genes that conditions that cell's properties. To ensure such selective gene expression, extensive networks of regulatory factors were developed, each protein having a peculiar program of transcription control.[2,3] In general, transcription regulation requires DNA sequence specific transcription factors (TF) that bind to gene regulatory sequences that may be the proximal promoter or the distal enhancers or silencers.

TF Composition

DNA sequence specific TF are modular proteins constituted by DNA binding and activator modules that are under the control of regulatory modules. The regulatory module may be an integral part of the TF molecule or alternatively, it is incorporated into the TF by independent regulatory events (for an example, IκB that regulates the activity of NF-κB family). There are many transcription factors, that belong to different families and each family has many members. Within each family, the DNA binding motif is relatively conserved. DNA binding motifs recognize relatively short DNA sequences and thus bind to DNA with a relatively low affinity and low specificity.

Transcription Requires Multiple TF Binding

Since TF bind to DNA with low affinity, the precise control of gene expression is usually achieved by the utilization of multiple recognition sites present in clusters in the gene regulatory elements. Moreover, the specificity of gene transcription is frequently ensured by combinatory events, i.e., by multiple and diverse TF binding to multiple sites within promoters/enhancers/silencers.

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Mode of Action of DNA Sequence Specific TF

One of the first roles of TF is to initiate the modifications of the chromatin structure, rendering the gene accessible to transcription. It was described that some TF can initiate this process directly because they are able to bind their DNA recognition structures even when these are present within condensed chromatin, i.e., packed into nucleosomes. It is usually believed that initiation of gene transcription requires the participation of at least one of these TF. Once chromatin remodeling is initiated, other TFs may also bind. The combined TFs then recruit coregulators of transcription to the transcription site. Co-regulators may be co-activators or corepressors have a major role in ensuring the cell/tissue specificity of transcription, since they can induce or silence gene expression. Many of these factors are subunits of proteins that modify chromatin structure, or are themselves enzymes that modify the acetylation, methylation, phosphorylation or ribosylation status of histones. Other coregulators interact with the transcription apparatus. The most universal cofactor that serves to transduce regulatory information between DNA specific TF and the transcription apparatus and is the modular complex known as Mediator.[4]

The Transcription Apparatus

Gene transcription is mediated by RNA polymerase II (RNAP II). While in prokaryotes promoter regions are recognized directly, in eukaryotes RNAP II requires the contribution of other accessory proteins that recognize the conserved TATA box and initiation sequences present in most protein coding genes. Thus the transcription apparatus in eukaryotes is a protein complex where RNAP II is associated with other proteins named general transcription factors-GTF. In contrast to other DNA sequence recognition TF, GTF are relatively few and are quite conserved.

Gene Expression Is Dependent of Complex Regulatory Events

The pathways of gene activation may be regulated at multiple stages and no general rules exist describing how each individual pathway is regulated in each particular circumstance. Besides, different classes of genes sharing some common pathways may even be regulated at different stages.[3] Regulation may be achieved through the synthesis, activation, deactivation, conditional nuclear localization or degradation of DNA sequence specific TF or other coregulators of transcription. Frequently, each component involved in gene transcription is itself dependent on its own pathway of induction/activation/deactivation. This complex multi-factorial dependency of gene transcription confers a stochastic component to gene expression/down-regulation.

The Gene Expression' Stochastic Component

Physicists swim happily in probability waters but in immunological terms, probabilities induce in immunologists reactions that go from mild rashes to severe anaphylaxis. However, one of the major aspects of cell biology i.e., the initiation of gene transcription was clearly shown to be stochastic. To study this aspect, single-cells were carefully selected to be totally homogeneous and they were submitted to the same stimulation conditions to induce gene expression.[5] A wide cell-to-cell variation was found. Individual cells started to express the same gene at different time points and expressed that gene at very different levels. This was shown to occur in bacteria, fungi and more recently in CD8 T cells responding to antigens in vivo.[6] Moreover, in conditions where transcription enhancers' effects were studied at single-cell-level it was shown that they do not necessarily induce transcription, they just act by increasing the probability of transcription.[7] This probabilistic component makes sense if one considers the multiple events that must concur to induce gene expression. Multiple transcription factors and regulatory proteins have to be present simultaneously to regulate transcription. Each of these factors is by itself dependent on its own peculiar pathway of activation and sometimes it is yet to be synthesized (what will in itself require other previous multifactor stochastic events...). It would be thus be unlikely all these requirements reach precisely the same level at precisely the same moment in each individual cell. Cell-to-cell variation is believed to have a major role in the diversification of an initially homogeneous cell population. An initial variation of gene expression may generate cells that do not respond to environmental stimuli exactly in the same way, what may induce further diversification, which

will also not be homogeneous. In this way, an initial homogeneous population may generate very different cell types through successive diversification steps. Although available studies only studied the induction of gene expression it is very likely that down-modulation of gene expression may also be influenced by similar rules.

Gene Expression Is Not an "All or Nothing Event"

Cells that do not express a gene may differ in the requirements necessary for that gene transcription. Common examples are cytokine genes in T-cell responses. While naïve cells require relatively long induction periods and stronger triggering conditions, memory cells rapidly secrete cytokines and do so in less favorable conditions of TCR stimulation. This phenomenon was found to be due to modifications of the accessibility of cytokine gene regulatory elements (that are induced during priming and maintained in memory cells) and is generally referred to as "induction of locus accessibility". However, this term lacks precision, because it refers to any type of epigenetic modification. It may involve several different and progressive events that may affect either the gene or any of its regulatory elements, the DNA or the histones' status and the spatial organization of the gene and its regulatory elements within the nucleus (for example, locus organization into transcriptional factories or chromosome territories).[8] Thus, loci are not just accessible or not accessible, they may be more or less accessible. The processes initiating loci accessibility may already occur during T-cell differentiation in the thymus well before mature T cells initiate immune responses. Once a gene is expressed, major variations may also be found in the rate of transcription, conditioned by either the cell' activation status, the stability of the transcription or the intrinsic properties of the gene.[6] Thus, during CD8 immune responses the number of Granzyme B (*Gzmb*) and IFN-γ (*Ifng*) mRNA molecules each cell expresses is much higher at the peak of the response than at other time points. When CD8 initiate the expression of a particular effector gene expression, individual cells show major cell-to-cell variations in the transcription rates of that gene while messages show little cell to cell variation once transcription becomes permanent. Finally, each gene has a peculiar transcription rate. For example, perforin (*Prf1*) transcription levels average 10^3 mRNA molecules/cell while and *Gzmb* transcripts average 10^6 mRNAs/cell.[6]

Epigenetic modifications were mostly characterized in CD4 Th1/Th2 polarization in vitro,[9] only sporadic reports studying CD8s in vitro[10,11] or in vivo.[12-15] The *Il2* and *Ifng* promoters' CpG motifs demethylate after CD8 activation and this process is more rapid in memory cells. H3 acetylation increases in the same loci. Interestingly, CD8 memory cells generated in the absence of CD4 help have significantly less histone acetylation than "helped" CD8s.[13]

The Gene Regulatory Elements Involved in CD8 Responses

Many gene transcription regulatory elements are known to be involved in CD8 differentiation and it is likely that many more will be eventually identified. However, some of them are known to be key factors, required for the acquisition of CD8 effector functions.

Major Regulators of CD8 Function

NFAT

The NFAT (nuclear factor of activated T cells) is an ubiquitous family of transcription factors comprising multiple members with different tissue representation. With the exception of NFAT5 (activated by osmotic stress), NFAT proteins integrate Ca^{2+} mediated signals in a large variety of eukaryotic cells.[16] The NFAT1 and NFAT2 are crucial for CD8 differentiation, NFAT1/NFAT2 double deficient cells being unable to produce several cytokines (IL-2, IFN-γ, TNF) and to exert cytolytic functions.[17] NFAT transcriptional activity is induced by TCR stimulation via a Ca^{2+} dependent calmodulin activation of calcineurin. This phosphatase dephosphorylates the NFAT's NLS, allowing NFAT nuclear translocation.[18] This process is reversible, regulated at multiple steps, the NFAT's long-term residence in the nucleus requiring sustained Ca^{2+} influx.[19,20]

NFATs usually act in concert with other TFs. The most common partners belong to the AP-1 TF family (usually Fos/Jun), which are themselves induced by other T-cell signaling pathways

(PKCθ, Ras, MAPKs).[21] The regulatory elements of several CD8 effector genes contain compound NFAT/AP-1 sites, this co-operative binding decreasing individual TF fall-off rates. Besides, each factor may act independently, NFAT cooperating with several other TFs activated by either the TCR, costimulation or cytokines/chemokines signaling but the structural details of these interactions are largely unknown.[22]

The role NFAT TFs in CD8 expression of effector genes is complex. Depending on the context NFAT family members may increase or decrease the expression of individual genes.[23]

T-bet and Eomesodermin (Eomes)

These factors belong to T-box family and have a highly homologous (74%) classic T-Box DNA-binding domain. They are not expressed in resting cells, but are rapidly induced after TCR stimulation. Signaling through the IFN-γ receptor via STAT-1 can also induce T-bet (encoded by *Tbx21*) expression. These two TFs have a fundamental role in the several events occurring during CD8 differentiation. Depending on each individual gene, each one of these TF may be either redundant or have a dominant role in that gene's expression. However, the study of these TF impact did not cover many of the genes expressed after CD8 activation that may be regulated otherwise.

Eomes was shown to interact directly with the *Il2rb* promoter and to be fundamental for IL-2Rβ up-regulation after CD8 activation[24] while T-bet has a modest role.[25] It is thus believed that Eomes is fundamental for the response of memory CD8 T cells to IL-15. Eomes is also dominant in influencing *Ifng*, *Prf1* and *Gzmb* transcription- *Eomes* $^{+/-}$ mice already showing defects in granzyme B and perforin expression, while the *Tbx21* deficiency has little, if any, impact in CD8' capacity to express these mRNAs. However T-bet also contributes to the transcription of these genes since *Eomes* $^{+/-}$ CD8 T cells lacking *Tbx21* have a severe defect in IFN-γ expression.[24] In contrast, T-bet was shown to have a dominant effect in promoting *Il12rb*[26] and *Cxcr3*[27] expression. Finally, T-bet deficiency was claimed to enforce a central memory phenotype and to correct the defects of CD8 function induced by the lack of CD4 help in LCMV responses.[28] However, these results are incompatible with other findings reporting incomplete protection to LCMV challenge in immunized *Tbx21*-deficient mice[29] and the major role of central memory cells in conferring protection to LCMV.

Other TF Reported to Play a Role in CD8 Responses

Several other TFs were described to be involved in CD8 differentiation. Signaling through TCR, CD28 or cytokines converges to NF-κB TF family members' activation. These TF recognize similar DNA motifs as NFAT proteins and homo- or hetero-dimers of different members (such as p50, p52, p65, c-Rel and RelB) determine the nature of the regulatory event. TCR activation phosphorylates Ets proteins, that among other effects, collaborate with T-bet to increase IFN-γ secretion in CD4 cells.[30] STAT (signal transducers and activators of transcription) family members mediate signals provided by various cytokines, binding to STAT DNA motifs in cytokine inducible genes. NFAT induced chromatin remodeling often occurs in cooperation with STATs.[22] The ubiquitously expressed SP-1 regulates the activity of many other TFs, including that of T-bet.[31,32] ATF/CREB are induced by TCR stimulation and also may act as transcriptional activators or repressors, depending on their binding partners.[33] Finally, Runx TFs were reported to be involved in CD8 division but it is unclear if this role is restricted to thymus differentiation or if it also affects mature T cells.[34]

Transcriptional Repressors (TR)

Several TR were shown to be involved in CD8 responses, some positively regulating the generation of long-term memory (Bcl6, Bcl6b and LKLF), while others having the opposite effect (Blimp-1, Id2). In most cases the mechanisms involved are unknown.

KLF2 (or LKLF, lung Kruppel-like factor)-deficient T cells show spontaneous activation, increased death rates and severe CD8 depletion.[35] KLF2 is expressed in naïve cells, rapidly down-regulated following activation and re-expressed in memory T cells.[36] It is upregulated by

cytokines IL-2 and IL-7 and KLF2 expression levels correlate to the survival of memory T cells in vitro and in vivo. KLF2 is believed to contribute to cell quiescence since the ectopic expression in Jurkat T cells reduces protein synthesis, cell size, proliferation and the expression of activation markers and up-regulates the expression of CD62L and S1P1.[37,38] Some of these effects may be due to down-regulation of c-Myc but further characterization is required to elucidate its role in T-cell differentiation.

Blimp-1 (B lymphocyte-induced maturation protein) deficient mice develop severe autoimmunity, but the contribution of different lymphocyte types to this syndrome is yet to be established.[39,40] Blimp-1 is largely absent in naïve cells, expressed at a high level in effector cells and at a lower level in memory cells.[41] It was shown to directly repress *Bcl6*, *Ifng*, *Il2*, *Tbx21*, *Fos* and *Myc* and it is proposed to suppress cell division and induce cell death.[40] Blimp-1 down-regulation of *Tbx21* and effector molecules suggests this factor blocks effector' differentiation, but how this contributes to memory generation is yet unclear.

Bcl6 deficient mice have no germinal centers, exhibit Th2 skewing and die from an inflammatory syndrome marked by myocarditis and pulmonary vasculitis with eosinophil infiltrations, again making it hard to distinguish its specific role in CD8 differentiation.[42,43] Bcl6 expression is induced upon T-cell activation but relative expression levels in effector versus memory cells were not detailed.[44] Bcl6 binds several corepressors, thereby recruiting histone deacetylases to the silencer regions of target genes. In particular, it was shown to associate directly with the *Gzmb* promoter and to suppress *Gzmb* expression in a reporter assay.[45] Overexpression of Bcl6 in T cells was initially reported to promote CD62L^{hi} generation and their proliferation in secondary responses[46] but different immunizations issued contradictory results.[44,46] A closely related TR to Bcl6 is Bcl6b (or BAZF) that was implicated in promoting the magnitude of CD8 secondary responses.[47] Bcl6b and Bcl6 bind an identical DNA motif (which is reminiscent to a STAT motif) and the repressive effect of Bcl6b was proposed to be mediated through association with Bcl6.[48,49]

Id2 (inhibitor of DNA binding-2) exerts its repressive effect by binding to E protein transcription factors, thus inhibiting their DNA binding.[50] Id2 is upregulated after Ag-stimulation and kept at stable levels in memory cells. *Id2*-deficient mice lack some subsets of T_{EM} CD8s. Moreover, during an immune response *Id2* KO CD8 cells expanded poorly, contracted rapidly and rapidly acquired a T_{CM} phenotype.[51]

Regulation of Individual Effector Genes

The expression of individual effector genes is regulated by extensive networks of TF (Table 1). *Ifng*, *Prf1*, *Gzmb* and *Fasl* in CD8 T cells were extensively studied. The TFs conditioning TGF-β gene *(Tgfb1)* transcription shown in Table 1 were characterized in epithelial cells or fibroblasts. Since different cell types may use different regulatory elements to control the expression of the same gene[52] *Tgfb1* data must be taken with caution because it may not apply to CD8 lymphocytes. For some effector genes little is known. T-bet associates to the promoters of *Ccl3* and *Ccl4* chemokines and T-bet overexpression studies showed *Ccl3* upregulation, but *Ccl4* regulation was not tested directly[25] and other regulatory elements were not characterized. KLF13 occurs rather late after naïve T-cell activation, binds to the *Ccl5* promoter and participates in the transcription of *Ccl5*, but KLF13 deficient cells yet express this gene, indicating redundancy. In human T cells *Gzma* and *Gzmb* are expressed in different CD8 populations,[53] but regulation of *Gzma* expression was not characterized.

References describing in further detail promoter and enhancer regions and individual regulatory elements already characterized to promote or silence each gene expression are quoted in Table 1. However, this Table rather reveals an important fact. The regulation of individual effector genes does not overlap. While some of the TF are involved in the regulation of several effector genes, others are not. Importantly, if one considers the combination of all regulatory elements, all individual genes differ. Since transcription regulation is determined by combinatory events, there is ample opportunity for diversity in the expression of each individual effector gene. Finally, this table only refers to effector genes, but during immune responses CD8 T cells also modify the expression of a

Table 1. Transcription factors known to regulate individual effector genes. The list only includes TF that were demonstrated both to bind the promoter regions and to influence transcription.

Target Gene	Activation	Repression	References
Tgfb1	AP-1; SMAD2, 3	AP-1; SP-1	54-57
Tnf	NFAT; SP-1; ATF2/cJun		58,59
Il2	NFAT; AP1; NF-κB; Oct	T-bet; Ikaros	60-62
Ifng	NFAT; T-bet; AP-1; NF-κB; Ets-1; ATF2/cJun; HMGA1; STAT1, 3, 4, 5; C/EBP; Runx3	ATF1/CREB; SMAD2,3; NF-κB (p50/p50), GATA3 and STAT6	63
Prf1	NFAT, T-bet; Eomes; AP-1, NF-κB, Ets-1, SP-1, MEF and STAT3, 4, 5		24,64
Gzmb	NFAT; T-bet; Eomes; AP-1; Ets-1; Runx1; Ikaros	ATF1; SMAD2, 3; Bcl6	24,64
Fasl	NFAT; AP-1; Ets-1; SP-1; Egr2, 3; Myc		64
Ccl5	KLF13; NFAT; NF-κB		65,66

Please note: These activities were sometimes tested in other cell lineages and may not be operable in CD8 cells (see text). Eomes was found to regulate *Ifng* expression, but binding activity was not yet demonstrated. The direct mechanisms of action of several repressors (such as Blimp-1, LKLF) are still poorly defined and were omitted from the table.

vast number of other molecules. If the expression of each of these molecules is also under peculiar regulation, the CD8 responses may yet show an unsuspected diversity.

Modifications of Gene Expression in CD8 Responses

Methodologies to Evaluate Gene Expression by CD8 T cells

Gene expression may be evaluated by two approach' types that give complementary but not overlapping information. In one type ("population studies") the total mRNA pool from each cell set is isolated. Then, the different mRNAs recovered from different cell sets are compared, to determine which genes are differentially expressed. The alternative approach scores transcription by individual cells ("single-cell studies"). Each of these methods has limitations of two types. The first is related to each technique's limitations that may yet be reduced with time. Importantly, there are other fundamental differences in the type of information that each method can provide, what is frequently overlooked and may lead to data over-interpretation and misconceptions in the field.

Population Studies

The most currently used strategy for "population studies" compares gene expression in two or more different populations by the use of gene expression arrays. This method has as major advantages that of being quite easy to perform and of covering a vast set of genes. However, arrays were not set up to study CD8 T cells in particular and thus probes with the capacity to detect genes that are "CD8 specific" may be underrepresented or not present at all, while positive signals may correspond to expressed sequence tag (EST) with yet unknown roles. "CD8 specific" cDNAs may be not very abundant either because transcription rates are low or/and because not all cells from that population transcribe that particular gene. Finally, "CD8 specific" cDNAs are put in contact with multiple probes, may have yet unknown cross-hybridization patterns and less-specific, low

affinity binding of cDNA to the multiple probes present in the array may "consume" less abundant cDNAs and "dump" specific signals. By these limitations, DNA arrays may fail to detect differentially expressed genes with an important role in CD8 differentiation. These inconveniences may be overcome by the more fastidious and technically demanding subtraction library technology that is yet the most accurate way to identify differentially expressed genes. The major advantage of subtraction libraries versus array studies is demonstrated in the identification of *Tbx21*. That TF that has a fundamental role in CD8 differentiation was identified in subtraction libraries.[67] In contrast, array studies comparing naïve and effector CD8 cells did not show a differential expression of these genes,[68] although *Tbx21*and *Eomes* are not expressed at all in naïve cells and are expressed by virtually all CD8 effectors.

Of major interest, comparison of various CD8 populations may reveal differentially expressed genes that were not previously identified as having any role in CD8 differentiation and thus become ideal candidates for further studies. Therefore, population studies are ideally approaches to "fish genes" with previously unknown functions.

Single-cell analysis is at the opposite end of population studies.[69] It is laborious and can only address the expression of known genes. The number of genes that can be studied simultaneously in each individual cell is actually restricted to about twenty. However, it gives fundamental information on how known genes are expressed within a population, that cannot be obtained by array studies. It determines the frequency of cells expressing each gene. It studies gene association. These two types of information are fundamental to understand the role of each gene within that cell set, to predict cell function and to determine a possible heterogeneity and sub-division of cells types. In contrast, in population studies the mRNA are pooled from many individual cells and thus evaluate the population's mRNA expression averages, what is frequently insufficient and may even be misleading. Figure 1 exemplifies potential misconceptions by comparing the same population (CD8 T cells recovered at 4 days after priming) studied simultaneously at a "population" or at "single-cell" level.

The Importance of Frequency Estimates

Individual genes are transcribed at very different levels, which are "characteristic" of each gene. Transcription can range from >10^7 mRNAs/cell (*Gzmb, Gzma*) to 10^3 mRNAs/cell (*Tgfb1*, *Prf1*).[6] Consequently, a single cell expressing *Gzmb* at 10^6 mRNAs/cell present at 1/1,000 frequency may give the same signal as 100% of the cells expressing *Tgfb1* at 10^3 mRNAs/cell, i.e., in "population" readouts a rare nonrepresentative event at 10^{-3} frequency and a major property shared by all T cells may score similarly. This major bias is evident in Figure 1. *Gzmb* was the most abundant gene expressed by the CD8 population, but single-cell analysis revealed that such signal was due to very rare cells expressing *Gzmb* at >10^6 copies/cell. In contrast, *Tgfb1* signal was much weaker but our single-cell analysis revealed that this gene was expressed by more than 70% of CD8s- at about 10^3 copies/cell. Single-cell read-outs are thus fundamental to determine the frequency of cells expressing each gene, i.e., how important is that property within that cell set.

Predicting Functional Behavior

The other major limitation of population studies is their inability to evaluate if different genes are co-expressed by the same cell or by different individual cells. This may be of importance if cell function requires the simultaneous expression of several molecules, as exemplified in Figure 1. In order to kill target cells efficiently each CD8 lymphocyte must co-express perforin and granzymes. CD8s studied at a population level appeared cytotoxic because both genes were detected but single-cell studies revealed that *Prf1* and *Gzmb* were usually expressed in different cells. These results suggested these cells were not cytotoxic, which we did confirm by in vivo functional tests. TGF-β may be anti-inflammatory by blocking T-cell proliferation or pro-inflammatory by mobilizing and activating APCs. Response to TGF-β requires the co-expression of two receptors by the same cell, one capturing the ligand and other responsible for signal transmission. Studies at population level detected the expression of *Tgfb1* and of both receptors, suggesting an anti-proliferation role, but single-cell studies revealed that T cells cannot respond to the TGF-β they produce, because

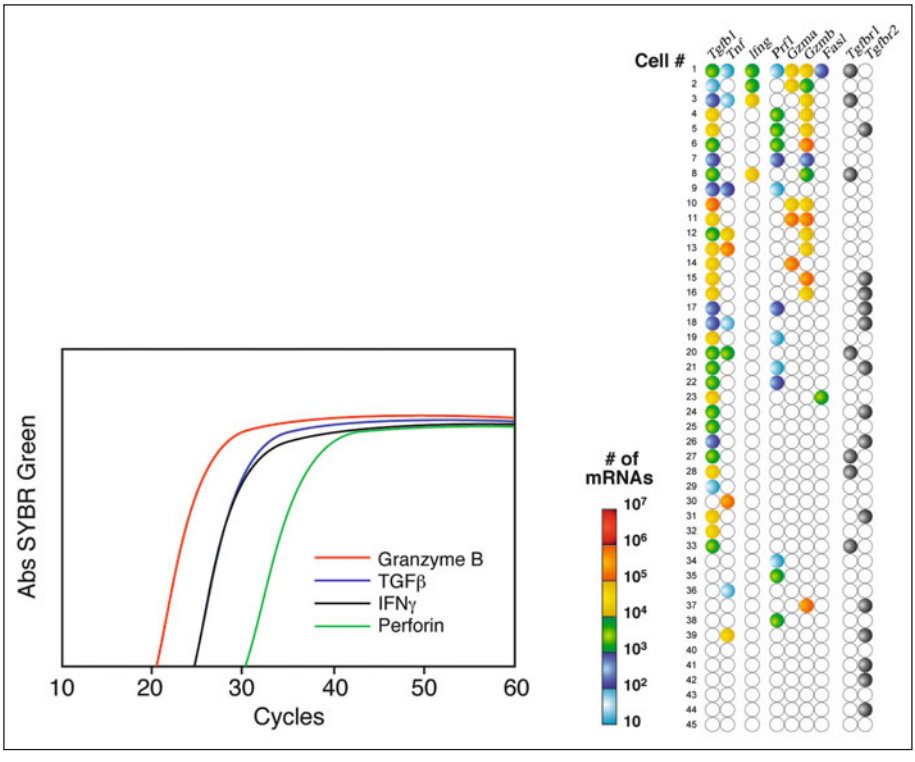

Figure 1. Population versus single-cell studies. Results compare gene expression levels of the same CD8 cell set that was isolated and studied simultaneously as a population (left) or as single cells (right). In single cell studies each row shows the same individual cell that is numbered. Each column shows a different gene representing the number of mRNA molecules/cell according to a colour scale. Empty symbols represent cells that do not express that gene (<2mRNA/cell) and grey symbols positive cells where gene expression levels were not quantified.

individual cells did not co-express the *Tgfbr1* and *Tgfbr2*.[6] This finding led us to envisage that cells expressing *Tgfb1* had a pro-inflammatory role of this cell set that we confirmed by in vivo functional studies.

Screening for New Subpopulations

Single-cell analysis allows the comparison of the co-expression of twenty different genes simultaneously. Thus it allows addressing how different effector molecules and different receptors described to be involved in CD8 responses all associate between themselves. It allows investigating if the CD8 population we are studying is homogeneous or constituted by different cell sub-types and in the later case, what are the receptors/effector molecules that define each sub-type. It must be noted that no other method allows the simultaneous comparison of twenty different parameters neither has the same sensitivity level. Flow cytometry has progressed but is yet very below 20 parameters evaluation and available conjugates frequently impose important restrictions to association studies. Moreover, in many circumstances single-cell co-expression of different genes cannot be evaluated at protein level since Abs recognizing native proteins in the mouse are not yet available (as in the case of perforin), or importantly, protein expression levels are so low they do not allow precise discrimination between positive/intermediate/negative cell types.

Thus, genetic arrays and single-cell analysis appear to have different complementary scopes. Genetic arrays are fundamental to identify potentially important genes that are differentially

expressed in two different cell sets. Single-cell analysis, by evaluating different genes expression frequencies and on their co-expression by the same cell gives important information on the importance of each gene within a cell set; indicates potentially different T-cell properties; may identify different T-cell subpopulations.

Differential Gene Expression during CD8 Responses

The transition of CD8 T cells from the naïve to the memory stage was investigated in gene expression arrays. For that purpose, LCMV specific P14 TCR-Tg populations were stimulated with LCMV and cells recovered at the response peak (classified as "effectors") were compared with naïve and memory cells, recovered at different time points after the contraction phase.[68,70] About 350 genes were differentially expressed, corresponding to 2-3% of the genes claimed to be represented in the array. This number is surprisingly low when compared to array studies performed in human T cells that shown the differential expression of 3,000 genes (17% of the genome) after in vitro stimulation.[71] Many of the differentially expressed signals corresponded to non-identified ESTs. Indeed, one of the signals showing important differential display between naïve and "effectors" and "effectors" and memory cells was the EST "moderately similar to the AF51064 from homo-sapiens".[68] Other differentially displayed genes gave predictable results. Thus dividing "effector" cells expressed higher levels of molecules involved in cell division, higher *Ifng* and *Gzms* and *Fasl*, multiple molecules involved in cell adhesion and migration as CD44, CD62L or Ly6C changes in the proteins involved signaling, including the previous described inversion of *Lck*/*Fyn* ratios[72] and the up-regulation of several molecules involved in Ca^{2+} or cytokine signaling. Not described before were the modifications of genes coding for the proteins involved in mitochondrial activity and protein translation that predict an increased metabolic capacity of "effector" cells. 30% of the genes differentially expressed by effector genes were modified in the "effector" to memory transition, but these modifications were not very revealing since they correlated to the diminished division rates, metabolic activity and effector molecules expression, or to previous described modifications of several surface markers as CD62L and Ly6C. However, this array studies reveled that after reaching steady-state numbers CD8 T memory cells are not necessarily stable populations, since their gene expression profiles keep on changing with time.

The Expression of Individual Effector Genes Throughout the Immune Response

Since individual effector genes are under the control of complex and diverse regulatory events (Table 1) it is not too unexpected that they do not behave similarly throughout the immune response. This was investigated in detail in the CD8 responses to the HY antigen and to *Listeria monocytogenes* (LM) expressing OVA.[6] These two responses were surprisingly similar although CD8 populations had different TCR specificities and responses were respectively to a minor transplantation antigen or to a bacterial infection.

CD8 cells were initially scored for 18 T-cell effector mRNAs and certain were excluded because they were not expressed (Th2 cytokines) or were very rare (*Il2*, *Il10*). Remaining effector molecules had different kinetics of expression and down-regulation and that did not necessarily correlate with CD8 accumulation/decay (Fig. 2). Thus, mRNAs coding for TGF-β and TNF-α were expressed immediately after activation. We later found out that mRNAs coding for the pro-inflammatory chemokines MIP-1α and MIP-1β were also expressed immediately after activation. Surprisingly, mRNA expression for TNF-α, MIP-1α and MIP-1β was very transient and was down regulated after the first divisions. In contrast, *Tgfb1* frequencies were maintained until the response peak. Although expression frequencies declined during contraction, a large fraction of memory cells yet express *Tgfb1*. CD8s present at the early phase of the response did not co-express *Prf1* and *Gzmb* or expressed *Fasl*.[6] These gene expression profiles suggested that that these cells could have a pro-inflammatory profile. Indeed, in vivo tests showed that these cells were unable to kill target cells, but rather mediated trapping and the local accumulation $CD45^+$ nonT nonB cells (presumably either DCs or/and monocytes/macrophages).

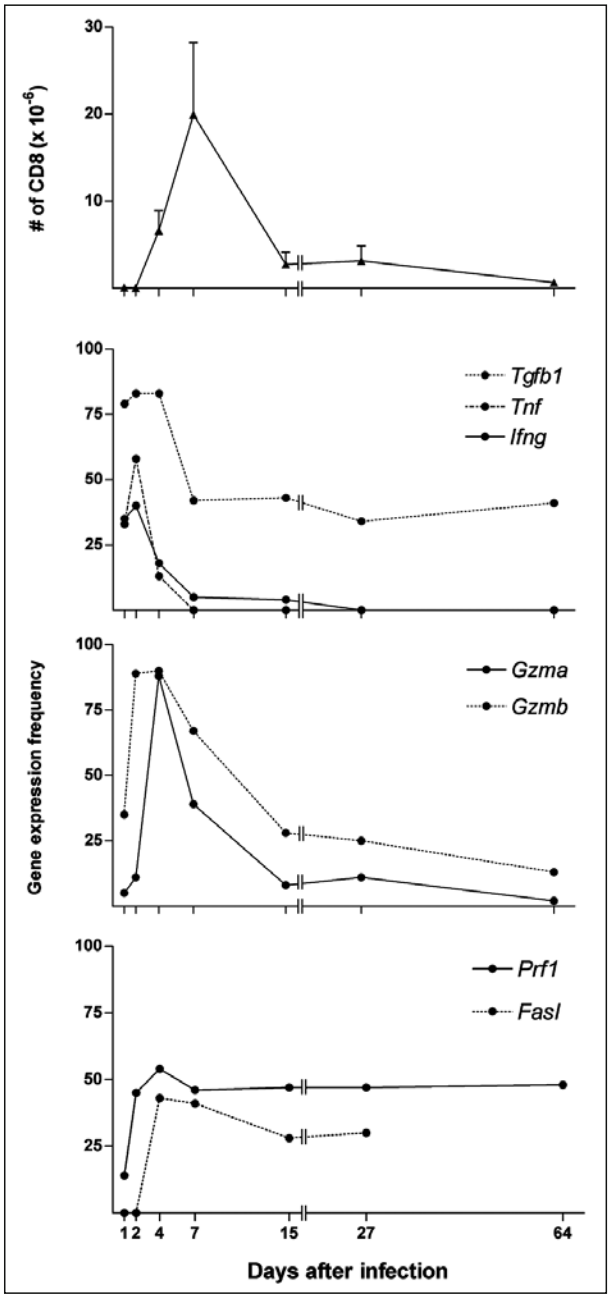

Figure 2. Kinetics of effector genes expression during the response to LM-OVA. OT-1 CD8 T cells were transferred to normal mice and immunized with LM-OVA. Results compare the kinetics of CD8 cell accumulation (upper graph) with the expression frequency of different effector genes. At days 1-2 not all CD8s were activated. Frequencies were determined in CD69+ cells, as other cells do not express effector mRNAs. *Ifng* and *Fasl* expression was not tested at day 64.

It thus appeared that CD8 T cells could become effector cells immediately after antigen encounter even before division, in contrast to the common notion that effectors are generated at the peak of the response. However, these effectors were not the classic killer cells. Rather, this initial "inflammatory" effector phase will have a major role in attracting innate immunity actors to site of the immune reaction. The generation of such "inflammatory effectors" in the anti-HY response also contradicts the common notion that inflammation is always the consequence of a nonspecific response to PAMPs expressed by pathogens. It shows that following antigen recognition, CD8 T cells can trigger an inflammatory reaction directly. This capacity to mobilize directly the innate immune system will be of major advantage to deal with tissue antigens, as those presented by tumor cells.

After this initial inflammatory phase, expanding cells progressively expressed other effector molecules in the following order: *Ifng*; *Prf1*; *Gzmb; Gzma;* and finally *Fasl* that was not detected at the beginning of the response. *Prf1* and *Fasl* expression frequencies were maintained constant throughout the response and were unchanged in memory cells. In contrast, other genes declined before CD8s reached the peak of the response. *Ifng* and *Gzma* down-regulation was very rapid, *Gzmb* decline was slower. Importantly, in vivo tests comparing the behavior of the same number of CD8 T cells present in the same location confirmed that killer function correlated to killer genes co-expression: early inflammatory effectors were unable to kill target cells and killer activity declined during contraction, when individual cells no longer co-expressed killer genes.

Interestingly, secondary responses followed different rules. When memory cells were re-stimulated in vivo, effector molecules became rapidly expressed. These results were expected, since priming is reported to induce "locus accessibility" that favors the rapid transcription of genes that are silent in resting memory cells. Surprisingly, this expression was maintained even after antigen elimination. Secondary "memory" cells co-express multiple effector genes, kill target cells much more efficiently than the primary memory cells and are even more efficient than the "cytotoxic effector cells" recovered at the peak of the primary immune reaction (Fig. 3). These properties of secondary memory cells are strictly dependent of CD4 help. This results lead to a surprising conclusion: After boosting secondary "memory" cells actually acquire the permanent functional

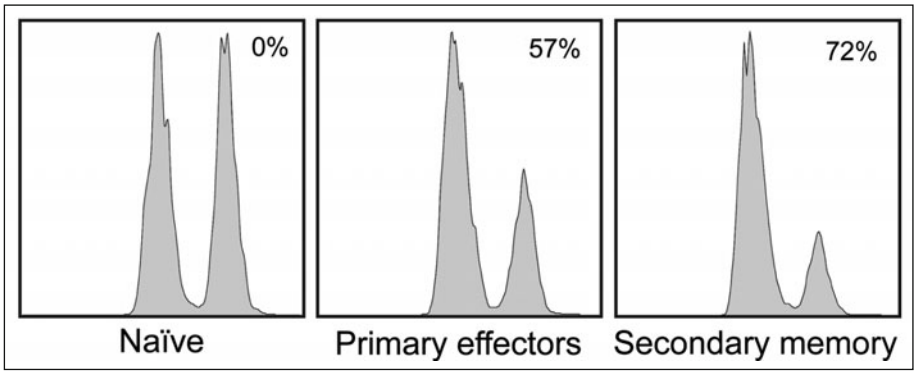

Figure 3. Boosting generates long-lived effectors. Results show in vivo killer activity of: left: naïve cells; middle: effector cells recovered at the peak of the primary response; right: secondary memory cells, obtained 3 months after the secondary immunization. In most cases where in vivo killing is investigated target cells are injected i.v. into the animal where they recirculate. Since it is very difficult to know how many killer cells are present in the whole mouse and where they meet target cells differences in target elimination can be due to differences in CD8 numbers/location or killer capacity. In these experiments CD8 populations were sorted. The same number was injected together with peptide-free $CFSE^{low}$ and peptide-loaded $CFSE^{high}$ B-target cells in spleen and killer activity determined after 4 h—what allows to determine killer capacity of the same number of CD8 cells meeting the same targets in the same location.

properties of effector cells, but, in contrast to "primary" effectors, they persist for long time periods in the absence of antigen.

These data raise several considerations that force us to shift the paradigms we currently apply to immune reactions. Firstly, CD8s effector gene transcription does not necessarily correlate to the presence or absence of the antigen, contradicting the notion that CD8s transcribe effector genes when the antigen is present and to stop their expression when antigen is eliminated. The transcription of pro-inflammatory chemokines and *Tnf* is down-regulated at the beginning of the immune response when antigen is yet abundant, suggesting yet unknown in vivo mechanisms constraining local inflammatory reactions. It is tempting to speculate the existence of other mechanisms restricting immune reactions by ensuring the rapid down-regulation of other receptors/effector molecules. For example, a rapid in vivo down-regulation of *Il2* and CD25 may explain why IL-2 is rarely detected in vivo[73] and many in vivo activated CD8 T cells do not express CD25 in spite of the importance of this cytokine and this receptor for CD8 in vivo growth and differentiation.[74] Other effector mRNAs persist for different periods in the primary reaction and moreover, became permanently transcribed in secondary responses. Thus effectors' transcription appears to be progressive. Successive antigen triggering (or perhaps other signals, for example cytokine mediated) may progressively modify effector gene loci eventually inducing antigen-independent, permanent and hereditary transcription. Secondly, we must modify several common concepts we use to define the steps/properties of T cells during immune reactions. We usually regard effector cells as short-lived cells generated at the peak of the response that do not survive long after antigen elimination. However, effectors may be present at all phases of the immune response. The detection of effectors with inflammatory functions emerging even before the beginning of the expansion phase demonstrates that differentiation into effector cells may precede cell division and occur well before the "official" effector phase, i.e., the peak of response. The presence of effector cells by the end of the secondary reaction (Fig. 2) shows that effectors are not necessarily short-lived, programmed to die rapidly. Like B cells that generate both short-lived and long-lived plasmocytes, CD8s may generate both short-lived and long-lived effectors that persist in vivo in the absence of antigen. Finally, the memory status is not a final differentiation stage. Memory cells change with successive antigen triggering. The final differentiation stage of the CD8 T cell may be rather the long lived antigen-independent effector cell that is so efficient to control antigen challenges that prevents even antigen accumulation as we and others found with secondary memory cells.[6,75,76] This may explain why vaccination frequently requires multiple antigen boosts to ensure efficient protection.

Different Infections May Induce Different Gene Expression Patterns

Considering the complexity of gene regulation it is not surprising that CD8 cells respond differently to various antigenic challenges. We observed major differences in gene expression patterns between in the LM response and the response to LCMV. These differences were not dependent on the TCR specificity. P14 Tg cells and T cells recognizing different LCMV epitopes all showed the same gene expression patterns. Besides, when P14 cells were immunized with LM-GP33, they behaved exactly like LM-OVA specific cells. This demonstrates that the gene expression patterns of CD8 T cells during immune responses are strongly influenced by the "infection" context.

Thus, several general rules established in the LM and HY response yet applied in LCMV. Individual effector genes had different kinetics of induction and down-regulation. Pro-inflammatory genes were expressed early on. In the contraction phase CD8 T cells lost *Prf1* and *Gzma/Gzmb* co-expression, correlating to their loss of killer capacity. However, the LCMV infection induced an immediate and overwhelming expression of *Ifng* that persisted well after infectious virus clearance, while *Ifng* expression in LM was rather transitory. In contrast, in LCMV *Tgfb1* was modest and very transitory while the majority of LM specific cells expressed *Tgfb1*and the expression of this gene persisted into memory phase. Major differences in the expression of several cytokine receptors were also detected- LM-specific cells express *Il10r*, *Il21r* and *Il12rb* while these receptors are absent or very rare in LCMV specific cells. Some of

these differences can be explained by previous findings but others suggest the presence of yet unknown regulatory pathways.

Thus, IL-12 up-regulates the expression of its receptor in Th1 cells in vitro.[77] A similar effect in CD8 T cells explains why during LM infection (where IL-12 is abundant)[78,79] all CD8 T cells express *Il12rb* at the response peak. TGF-β suppresses the expression of IFN-γ, justifying the rapid down-regulation of this cytokine found during the LM infection.[80,81] However, the differential expression of *Tgfb1* in LM and LCMV remains unexplained. It is yet unknown if IL-12 has any role in inducing *Tgfb1* or, alternatively, if IFN-α (that is produced in LCMV) mediates *Tgfb1* down-regulation. It is yet unclear if IFN-α has any role in favoring the persistent expression of IFN-γ during LCMV infection since this cytokine was described to have a modest in vitro effect. Finally *Eomes* and *Tbx21* are co-expressed by all LM-specific effector cells while many LCMV specific cells only express *Tbx21* and the reasons for this different expression are also unknown.

The Identification of CD8 Subpopulations: Man and Mouse Differ

One of the major advantages of single-cell multiparameter analysis was the possible identification of CD8 subtypes. Thus, one could hope that association of effector molecules expression with either CCR7, IL-7R, KLRG1 etc, could perhaps identify memory types with different characteristics, but this was not the case in the mouse.[6] In mouse memory cells, all molecules so far studied associated randomly between themselves and with any other of the effector genes/receptors expressed at the memory stage. In contrast, the cell surface markers CCR7 and CD45RA[82] together with CD27 and CD28[53,83-85] can subdivide human CD8 T cells into well-defined subtypes that show unique properties. Several aspects of these subtypes are remarkable. Firstly, each subset is quite homogeneous even when studied at single-cell-level: all individual cells having very similar or totally identical effector gene expression. Second, each subtype is characterized by the expression of restricted set of effector genes. Finally, these properties are the same in different healthy donors. This is rather surprising, considering the major variation usually found in most human studies. However, variation is only found in the relative representation of each subset. The percentage of cells belonging to each subset varies. The properties of each subset are remarkably similar even when single cells are studied.[53] Mouse CD8 T cells do not express some surface markers expressed by human cells and when markers are expressed, they do not subdivide equivalent subsets.

Thus, naïve T cells are CD45RA$^+$ CCR7$^+$, co-expressing IL-7R, CD27, CD28 and CD62L.[53,82,85] They do not express any effector gene.[53,85] Central memory cells (T_{CM}) are CD45RA$^-$ CCR7^{low} and maintain the expression of IL-7R, CD27, CD28 and CD62L.[82] All express *Ccl5*, but no other effector molecules. Remaining memory cells with a CCR7$^-$ phenotype are globally named effector memory cells (T_{EM}) but they are actually a complex mixture of CD8 cell types, that can be subdivided by their CD27/28 phenotype into discrete subtypes with a progressive differentiation status.[53,83,85] In their less differentiated state T_{EM}, are rather similar to $T_{CM.}$ They are CD27^{hi} and cells with this phenotype also express IL-7R and CD62L. The expression of *Gzma* defines this cell set since besides *Ccl5* (that is already expressed by T_{CM}), CD27$^+$ CD28$^+$ T_{EM} also express *Gzma* and *Prf1* but no other molecules involved in cell cytotoxicity. In their most differentiated stage T_{EM} are CD27$^-$ CD28$^-$ and do not express CD62L and *Il7r*.[53,85] All cells co-express *Prf1* and *Gzma*. *Gzmb* is abundant and some cells also express *Ifng*. The CD27$^+$ CD28$^-$ intermediate population is the most heterogeneous. It harbors both *Il7r*$^+$ and *Il7r*$^-$ subtypes, CD62L down-regulates but some cells yet expressed it. Besides *Ccl5* and *Gzma* some cells also express *Prf1* and rarely *Gzmb.*

Human cells also differ in the type of effector genes more frequently expressed. *Gzma* (rather than *Gzmb*) is the dominant granzyme expressed by human T cells. The very frequent expression of this gene as well as its expression in the absence of any other cytotoxic molecules (as found in CD27$^+$CD28$^+$cells) appears to be characteristic of human CD8 T cells. Finally, *Fasl* and *Ifng* are rarely expressed.[53]

Conclusion

Human thought processes can most easily visualize complex processes as a linear series of events, each step going to completion before the next step begins. Thus, based on the kinetics of T-cell accumulation CD8 T-cell responses are commonly subdivided in successive phases generally overviewed as independent: 1) the "expansion phase" where T cells are viewed as dividing only; (2) the response peak/plateau/effector phase, where T cells are thought as effector cells eliminating the antigen; (3) The contraction phase, where cells are looked at as mostly dying; (4) and the memory phase where they are thought to lose effector functions and revert to a resting state. The above data shows this "stepwise" perspective (that influences the field) is far from the truth. When all parameters are taken into consideration, the kinetics of CD8 accumulation/decay does not discriminate discrete differentiation stages. While all CD8 responses go through expansion/plateau/contraction/memory phases, the sole word that appears to define the modifications of gene expression is variability. Individual cells behave differently. Different genes are expressed/down-regulated with different kinetics. The infection context influences which genes are predominantly expressed and which continue to be expressed in memory cells. Finally, successive boosting leads to the generation of long-lived cells co-expressing multiple effector molecules. In general it thus appears that CD8 T cells may progressively evolve in their capacity to transcribe effector genes to reach a stage of permanent hereditary transcription. It remains to be evaluated how transcription rates reflect protein expression levels. In most cases this was not studied in sufficient detail and important limitations yet hinder protein evaluation. We do not dispose of any amplification methods to detect proteins that are equivalent in efficiency to those available for mRNA expression studies. Most proteins are expressed at low levels, difficult to discriminate from background values. Intracellular proteins segregating into peculiar compartments can be better evaluated by confocal microscopy but immunologists do not use this method systematically. Many antibodies are generated from extracted proteins that no longer maintain in vivo configurations and frequently they do not recognize native forms. Finally many proteins are rapidly secreted and cannot be visualized ex vivo. Therefore, current methods yet fail to detect a major fraction of cellular proteins at a single-cell-level and several examples of these limitations can be quoted. It was not yet possible to detect IL-2 in vivo, but many studies using IL-2 deficient mice revealed the importance of this molecule in immune responses in general and in CD8 differentiation in particular.[74] Memory cells express *Ccl5* mRNA.[86,87] It was claimed that this mRNA was not translated in mouse memory cells, but it was later shown that RANTES protein was accumulated in small granules in human memory T cells.[88] Thus, with respect to proteins, it is important to conclude that negative results are not conclusive.

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Chapter 3

The Role of Interleukin-2 in Memory CD8 Cell Differentiation

Onur Boyman,* Jae-Ho Cho and Jonathan Sprent

Abstract

The current literature on the role of interleukin (IL)-2 in memory CD8$^+$ T-cell differentiation indicates a significant contribution of IL-2 during primary and also secondary expansion of CD8$^+$ T cells. IL-2 seems to be responsible for optimal expansion and generation of effector functions following primary antigenic challenge. As the magnitude of T-cell expansion determines the numbers of memory CD8$^+$ T cells surviving after pathogen elimination, these events influence memory cell generation. Moreover, during the contraction phase of an immune response where most antigen-specific CD8$^+$ T cells disappear by apoptosis, IL-2 signals are able to rescue CD8$^+$ T cells from cell death and provide a durable increase in memory CD8$^+$ T-cell counts. At the memory stage, CD8$^+$ T-cell frequencies can be boosted by administration of exogenous IL-2. Significantly, only CD8$^+$ T cells that have received IL-2 signals during initial priming are able to mediate efficient secondary expansion following renewed antigenic challenge. Thus, IL-2 signals during different phases of an immune response are key in optimizing CD8$^+$ T-cell functions, thereby affecting both primary and secondary responses of these T cells.

Introduction

Typical T-cell receptor (TCR)αβ T cells are derived from precursors that migrate to the thymus where they undergo a series of selection and maturation processes termed positive and negative selection.[1] During positive selection, T cells with newly-arranged TCRαβ complexes are tested for their ability to receive survival signals via TCR contact with self-peptides bound to major histocompatibility complex (MHC) molecules expressed on cortical epithelium: cells with low but significant reactivity for self-peptide/MHC ligands are selected for survival while low-affinity cells die in situ, thus selecting only T cells with functional TCRs. Conversely, through contact with antigen on bone marrow (BM)-derived dendritic cells (DCs), negative selection eliminates T cells with high affinity for self-peptide/MHC molecules, thus ensuring the deletion of potentially auto-reactive T cells. At the end of these selection processes, mature CD4$^+$ and CD8$^+$ T cells are generated and subsequently released into the bloodstream for export to the secondary lymphoid organs. Despite the fact that these cells are selected on self-peptide/MHC molecules in the thymus, the cells have not yet seen foreign antigens and are thus immunologically naïve.

Post-thymic naïve T cells recirculate continuously between blood and lymph through the lymphoid tissues and remain in interphase, rarely if ever dividing.[2,3] Activation of naïve T cells occurs in the secondary lymphoid organs, such as lymph nodes and spleen, upon encounter with their cognate antigen in the form of peptides bound to MHC molecules presented by mature

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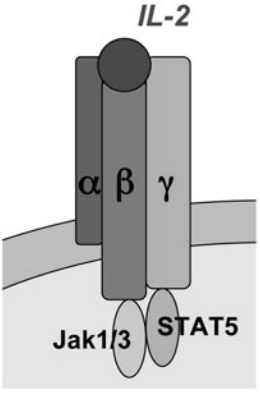
antigen-presenting cells (APCs) that express costimulatory molecules, notably DCs.[4] Upon activation, T cells undergo vigorous clonal expansion and differentiate into effector cells which then home to the site of infection. These primed cells can directly exert their effector functions upon TCR engagement without the necessity for costimulatory signals; effector $CD8^+$ cells kill pathogen-infected cells whereas activated $CD4^+$ cells provide "help" for $CD8^+$ cell differentiation or induce B-cells to produce high-affinity antibodies.

At the end of the expansion of antigen-specific T cells, which usually occurs after the pathogen has been eliminated, the immune response undergoes a contraction phase where most antigen-specific effector T cells die via apoptosis.[5,6] However, a minority (about 5%) of antigen-specific T cells survive to become long-lived memory cells.[7,8] These cells are resting cells but, unlike naïve T cells, memory cells display certain surface markers (such as a high density of CD44 in mice) which distinguish these cells from naïve T cells. Notably, unmanipulated normal mice contain significant numbers of cells with high expression of CD44 ($CD44^{hi}$), thus closely resembling memory T cells found after deliberate antigen priming.[8,9] These $CD44^{hi}$ "memory-phenotype" (MP) cells account for about 10-15% of total T cells in young mice but become a majority population in old age; MP cells are thought to represent the descendents of T cells reacting to ubiquitous environmental or self-antigens.[10]

Both naïve and memory T cells are maintained in fairly stable numbers during normal steady-state conditions (reviewed in ref. 9). The homeostatic processes that govern T-cell survival are complex, but contact with two cytokines, IL-7 and IL-15, with or without TCR signals from contact with self-peptide/MHC ligands are of particular importance. For naïve T cells, these cells are maintained through constant low-level signals via contact with IL-7 and self-peptide/MHC molecules (MHC-I for $CD8^+$ and MHC-II for $CD4^+$ cells).[11,12] For most memory (and MP) $CD8^+$ T cells, by contrast, homeostasis depends on contact with both IL-7 and IL-15, while TCR contact with self-peptide/MHC-I ligands is relatively unimportant.[13-15] Similar to their $CD8^+$ counterparts, memory $CD4^+$ cells also require signals from IL-7 and IL-15 and do not depend on contact with MHC (MHC-II) molecules.[16-18] Typical memory and MP cells are resting cells which divide intermittently through contact with IL-15, the density of CD122, the receptor for IL-15, being higher on memory cells than naïve cells.[19] It should be mentioned that about one-third of MP cells are activated cells; these cells ignore cytokines and seem to be engaged in chronic responses to unknown self-peptide/MHC ligands, both for $CD4^+$ and $CD8^+$ cells.[20,21]

IL-7 and IL-15 belong to the family of common gamma chain (γ_c) cytokines, which share usage of the γ_c receptor (also called CD132). This family also includes another cytokine that plays a central role in T-cell homeostasis, namely IL-2. This cytokine exerts complex effects on typical mature T cells and is also primarily responsible for the survival of $CD4^+$ $CD25^+$ T regulatory cells (Tregs).[22] IL-2 is a 15 kDa short-chain four α-helical bundle cytokine and is produced mainly by activated $CD4^+$ T helper cells, although activated $CD8^+$ T cells, natural killer (NK) cells, NK T cells and DCs stimulated with microbial products are also able to secrete IL-2, albeit in low amounts.[23-29] IL-2 acts in an autocrine or paracrine fashion by binding to IL-2 receptors (IL-2Rs).[30] High-affinity IL-2Rs are trimeric receptors consisting of IL-2Rα (CD25), IL-2Rβ (CD122) and the γ_c chain (Fig. 1); these receptors bind strongly to IL-2 with a dissociation constant (K_d) of about 10^{-11} M.[31-33] Trimeric IL-2Rs are found on Tregs as well as on recently-activated normal T cells.[30,34] In addition to trimeric IL-2Rs, IL-2 can also bind to dimeric IL-2Rs consisting of CD122 and γ_c, albeit with a 100-fold lower affinity ($K_d \sim 10^{-9}$ M). Dimeric IL-2Rs bind IL-15 in addition to IL-2 (Fig. 1) and are found at high levels on resting memory and MP $CD8^+$ cells as well as NK cells and at low but significant levels on naïve $CD8^+$ cells. Notably, CD122 and γ_c are responsible for mediating intracellular signaling whereas CD25 confers high-affinity binding to IL-2 but does not directly contribute to signal transduction.[31]

Below, we will review the role of IL-2 in $CD8^+$ T-cell responses in vivo; the in vitro actions of IL-2 have been reviewed extensively elsewhere.[30-33] In particular, we will discuss the contribution of IL-2 to the different phases of a $CD8^+$ T-cell response, starting with $CD8^+$ cell activation and expansion, followed by the contraction phase and then the memory phase. During each of these different stages, IL-2 has a decisive effect on $CD8^+$ cells. Thus, by controlling initial T-cell expansion



	Low-affinity IL-2R (K_d 10^{-9} M)		
	High-affinity IL-2R (K_d 10^{-11} M)		
IL-2R subunit:	**α = IL-2Rα = CD25**	**β = IL-2Rβ = CD122**	**γ = γ_c = CD132**
Signal transduction:	**No signaling**	**Signaling**	**Signaling**
Other cytokines binding R subunit:	**None**	**IL-15**	**IL-4, IL-7, IL-9, IL-15, IL-21**

Figure 1. The IL-2 receptor (IL-2R) and its subunits. IL-2Rs are either dimeric IL-2Rαβ and bind IL-2 with a low affinity (Kd ~10^{-9} M) or trimeric IL-2Rαβγ and associate with IL-2 with a Kd of about 10^{-11} M. IL-2Rα (α subunit), also called CD25, is the private α chain of IL-2 and does not bind any other cytokine. Moreover, CD25 does not contain a cytoplasmic tail and thus is not involved in signaling. Conversely, IL-2Rβ (β subunit, also called CD122) and IL-2Rγ (γ subunit, also known as the common gamma chain, γ_c, or CD132) are crucial for signal transduction upon IL-2 binding to the IL-2R. CD122 is also a receptor subunit of the IL-15R, whereas γ_c is shared by all γ_c cytokines, i.e., IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Downstream signaling is mediated by the Jak-STAT pathway, notably involving Jak1, Jak3 and STAT5 as well as STAT3.

and differentiation during the primary response, IL-2 influences both the numbers and functions of the cells that survive to become long-lived memory cells.[35]

IL-2 Signals during Priming Lead to Qualitative and Quantitative Differences in CD8⁺ T-Cell Responses

Once naïve T cells encounter their cognate antigen presented by mature APCs and receive TCR and costimulatory signals, they become activated and begin to proliferate. Activation and proliferation of T cells induces many changes, including the upregulation of CD25 and CD122, thus leading to expression of trimeric high-affinity IL-2Rs (Fig. 2). At the same time, activated T cells, especially $CD4^+$ cells, start producing IL-2. Via synthesis of high-affinity IL-2Rs, activated T cells, including $CD8^+$ cells, are highly sensitive to IL-2.

The contribution of IL-2 signals to primary $CD8^+$ T-cell responses has been studied using IL-2- or IL-2R-deficient mice (Table 1). Activation, expansion and primary effector functions of $CD8^+$ T cells were tested in IL-$2^{-/-}$ mice following infection with lymphocytic choriomeningitis virus (LCMV) and vaccinia virus and also after peptide immunization or exposure to alloantigens.[36-41] The overall conclusion from these experiments is that functional immune responses do occur in

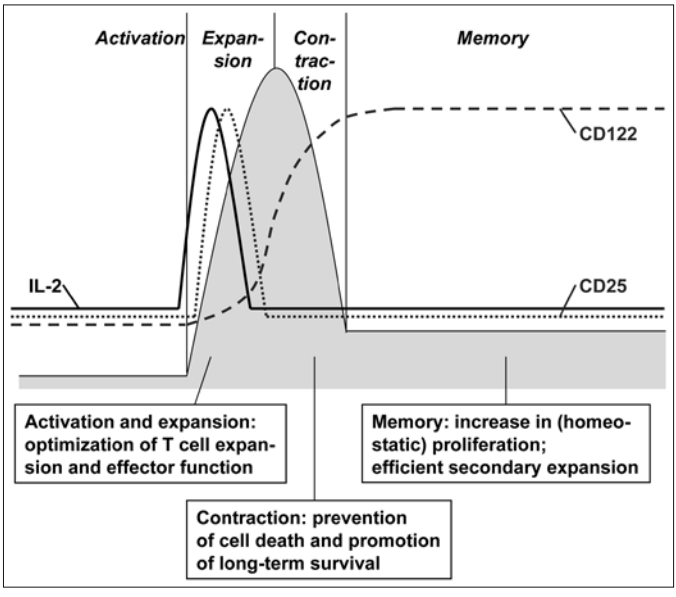


Figure 2. Expression levels of IL-2 and IL-2R subunits and the role of IL-2 during the different phases of a $CD8^+$ T-cell immune response. Following activation by a professional APC, naïve $CD8^+$ T cells start to proliferate and expand. Proliferation is considerably enhanced by the concomitant production of IL-2 (solid line), which initially binds to low-affinity IL-2Rαβ receptors and, upon upregulation of CD25 (dotted line), to high-affinity IL-2Rαβγ receptors. Notably, CD122 (dashed line) is also upregulated during expansion and is highest on memory T cells, where it serves mainly for conferring responsiveness to IL-15. The role of endogenous IL-2 during activation and proliferation of $CD8^+$ T cells is to optimize cell expansion; by contrast, contact with exogenous IL-2 during this phase does not seem to be necessary or beneficial. During the contraction phase, however, administration of exogenous (recombinant) IL-2 is highly beneficial in preventing cell death and thus allowing higher numbers of antigen-specific T cells to survive as memory cells for several months. Endogenous IL-2 seems to be dispensable for $CD8^+$ T-cell survival during the contraction phase. For memory $CD8^+$ T cells, homeostatic proliferation of these cells is augmented by the presence of endogenous IL-2 or the administration of exogenous IL-2.

$IL\text{-}2^{-/-}$ mice, but these responses are somewhat lower and less robust than in normal mice. The results are as follows; the shortcoming of the use of IL-2-deficient mice is discussed later.

In comparison to $IL\text{-}2^{+/-}$ heterozygous or wild-type (WT) control mice, $IL\text{-}2^{-/-}$ animals gave near-normal $CD8^+$ T-cell effector responses as measured by direct ex vivo cytotoxicity upon infection with vaccinia virus.[36,37] For infection with LCMV, a 3-fold decrease of cytotoxic T-lymphocyte (CTL) activity was noted in comparison to control mice when spleen cells from $IL\text{-}2^{-/-}$ mice were tested in a direct ex vivo cytotoxicity assay.[36-39] Moreover, spleen cells from $IL\text{-}2^{-/-}$ mice produced markedly reduced interferon (IFN)-γ and IL-4 levels upon in vitro restimulation for 24 h.[38,39] Two studies concluded that these differences were not biologically significant because $IL\text{-}2^{-/-}$ mice infected with LCMV were as efficient as $IL\text{-}2^{+/-}$ or WT mice in mounting a delayed-type hypersensitivity response (as measure by footpad-swelling reaction), clearing LCMV below detection levels from spleens, livers and kidneys by days 9-10 and protection against lethal choriomeningitis after intracerebral infection with LCMV.[36,37] Conversely, others came to a different conclusion, reporting that $IL\text{-}2^{-/-}$ mice infected with LCMV contained detectable virus in the spleen and especially in the kidneys on day 7 after infection, whereas IL-2-competent control mice had cleared LCMV from these organs by that time.[38] These disparate findings may reflect the different doses of LCMV and routes of administration (300 plaque-forming units (PFU) intravenously vs. 2000 PFU intraperitoneally, see Table 1) used

Table 1. Comparison of different studies examining the role of IL-2 during the different phases of $CD8^+$ T-cell immune responses

	Pathogen	Dose, Route	In Vivo Results and Outcome	Ref.
a) Activation, expansion and effector function during the primary response				
$IL\text{-}2^{-/-}$	VV WR	2×10^6 PFU i.v.	No difference in ex vivo cytotoxic response	36,37
$IL\text{-}2^{-/-}$	LCMV WE or LCMV Arm	2×10^2 PFU i.v. or 3×10^2 PFU i.v.	3-fold decreased frequency after expansion but equal virus clearance from spleen, liver and kidneys	36,37
$IL\text{-}2^{-/-}$	LCMV Arm	2×10^4 PFU i.p.	3-fold decreased frequency after expansion and reduced virus clearance from spleen and kidneys	38,39
OT-I $IL\text{-}2^{-/-}$ or OT-I $CD25^{-/-}$	VSV-Ova Ova EL4/E.G7	1×10^6 PFU i.v. 0.5-5 mg i.p. 5×10^6 i.d.	Decreased expansion in nonlymphoid organs	42,43
$IL\text{-}2^{-/-}$	Alloantigen		Allograft rejection associated with delayed or similar kinetics	40,41
$CD25^{-/-}$	LCMV Arm or LM-Ova	2×10^5 PFU i.p. or 3×10^3 PFU i.v.	Equal or 5-fold reduced expansion of $CD25^{-/-}$ $CD8^+$ cells	46,56
$CD122^{-/-}$	LCMV Arm	2×10^3 PFU i.v.	Defective primary $CD8^+$ cell response as measured by footpad swelling	55
$CD122^{-/-}$ tg	vJS510 # Agonistic anti-CD3 mAb	5×10^7 PFU i.p.	Signals through CD122 dispensable for generating CTL activity but necessary for optimal IFN-γ production in vivo	57-59
rhIL-2*	LCMV Arm	2×10^5 PFU i.p.	Equal expansion and virus clearance if rhIL-2 is given on days 0-8 after infection	49
rmIL-2	Alloantigen		Administration of IL-2 during first 4 d increases antigen-specific cell counts by 40%	60
IL-2	Superantigen		Prolonged expansion and survival of superantigen-specific T cells	61
b) Contraction phase				
$CD25^{-/-}$#	LCMV Arm or LM-Ova	2×10^5 PFU i.p. or 3×10^3 PFU i.v.	Similar contraction of $CD25^{-/-}$ and WT $CD8^+$ cells	46,56

continued on next page

Table 1. Continued

	Pathogen	Dose, Route	In Vivo Results and Outcome	Ref.
OT-I IL-2$^{-/-}$	VSV-Ova	1 x 10^6 PFU i.v.	Decreased apoptosis of antigen-specific T cells	42,59
rhIL-2*	LCMV Arm	2 x 10^5 PFU i.p.	Decreased apoptosis and increased long-term survival if rhIL-2 is given on days 8-15	49
c) Early and late memory, secondary responses				
IL-2$^{-/-}$	VV WR	2 x 10^6 PFU i.v.	Decreased secondary ex vivo cytotoxic response	36
IL-2$^{-/-}$	LCMV WE or LCMV Arm	2 x 10^2 PFU i.v. or 3 x 10^2 PFU i.v.	Equal footpad-swelling reaction and equal protection upon lethal challenge in vivo	36
CD25$^{-/-}$#	LCMV Arm or LM-Ova	2 x 10^5 PFU i.p. or 3 x 10^3 PFU i.v.	Impaired expansion of CD25$^{-/-}$ memory CD8$^+$ T cells due to decreased IL-2 signals during priming	46,56
rhIL-2*	LCMV Arm	2 x 10^5 PFU i.p.	Increased turnover and survival in IL-2-treated animals	49
d) Homeostasis				
CD25$^{-/-}$#	LCMV Arm	2 x 10^5 PFU i.p.	Equal survival of WT and CD25$^{-/-}$ CD8$^+$ cells	46
rhIL-2*	LCMV Arm	2 x 10^5 PFU i.p.	Increased turnover and survival of CD8$^+$ cells in IL-2-treated animals	49

Abbreviations and explanations: *: 15,000 international units (IU) rhIL-2 given twice daily; #: BM chimeras in which irradiated WT mice were reconstituted with T-cell-depleted BM from WT and CD25$^{-/-}$ mice; †: transgenic mice that express CD122 in thymocytes but lack CD122 expression in mature peripheral lymphocytes due to genetic deficiency; #: recombinant vaccinia virus expressing the spike protein S510 of the JHM strain of mouse hepatitis virus; VV WR: Vaccinia Virus strain Western Reserve; LCMV: lymphocytic choriomeningitis virus; WE: LCMV strain WE; Arm: LCMV strain Armstrong; Ova: ovalbumin; EL4 or E.G7: ovalbumin expressing tumor cell lines; VSV-Ova: recombinant vesicular stomatitis virus expressing ovalbumin; LM-Ova: recombinant Listeria monocytogenes expressing ovalbumin; PFU: plaque-forming units; i.v.: intravenous; i.p.: intraperitoneal; i.d.: intradermal; rhIL-2: recombinant human IL-2; rmIL-2: recombinant mouse IL-2.

in these studies. Whether this roughly 7-fold difference in initial viral load along with the dissimilar routes of infection could explain the differences observed remains to be tested.

Others found that IL-2 signals affected the expansion of antigen-specific CD8$^+$ T cells only in nonlymphoid tissues (such as in lamina propria, epithelia, liver and lungs) but not in lymphoid organs such as the spleen. Thus, after adoptive transfer of IL-2$^{-/-}$ ovalbumin-specific OT-I TCR transgenic (tg) CD8$^+$ T cells to IL-2$^{-/-}$ vs. WT mice followed by subsequent infection with recombinant vesicular stomatitis virus expressing ovalbumin, the authors found that paracrine IL-2 signals significantly increased the survival and sustained expansion of antigen-specific CD8$^+$ T cells in nonlymphoid tissues but not in spleen; paradoxically, autocrine IL-2 signals (observed with WT tg cells) negatively influenced expansion in nonlymphoid tissues.[42,43] In another study, Listeria monocytogenes (LM)-specific TCR tg CD8$^+$ T cells transferred to WT hosts underwent comparable expansion and production of IFN-γ after infection with LM regardless of whether the donor antigen-specific CD8$^+$ T cells were from an IL-2$^{-/-}$ or WT genetic background, thus providing further evidence that autocrine IL-2 was not essential for in vivo CD8$^+$ cell expansion and IFN-γ production in response to LM.[44] However, the responding CD8$^+$ T cells were still able to receive paracrine IL-2 signals in both situations.

Besides these above-mentioned studies on viral and bacterial infections, IL-2$^{-/-}$ mice were also used to test the role of IL-2 in CD8$^+$ responses to alloantigens. In one study, IL-2$^{-/-}$ vs. IL-2$^{+/-}$ and WT mice were rendered diabetic before transplantation with allogeneic islets grafts; allograft function was then followed by monitoring blood glucose measurements. The results showed that IL-2$^{-/-}$ mice were able to reject islet allografts, albeit with delayed kinetics compared to IL-2$^{+/-}$ or WT mice.[40] Similarly, in another study, vascularized cardiac allografts were rejected by IL-2$^{-/-}$ mice, though here rejection was as rapid as with WT mice.[41]

The contribution of IL-2 to CD8$^+$ T-cell responses has also been tested with the aid of CD25$^{-/-}$ mice. In the absence of CD25, IL-2 is able to bind to and signal by the dimeric IL-2Rβγ receptor,[45,46] even though such binding is around 100-fold weaker than to the trimeric high-affinity IL-2Rαβγ complexes.[31-33] To examine the influence of CD25, CD25$^{-/-}$ vs. WT OT-I TCR tg CD8$^+$ T cells were transferred to WT recipients, which then received either recombinant vesicular stomatitis virus expressing ovalbumin, soluble ovalbumin or tumor cells expressing ovalbumin. These experiments showed that IL-2 signaling through high-affinity IL-2Rs was not important for initial division of the responding CD8$^+$ cells, even though CD25 was upregulated on WT OT-I cells before the first division; however, IL-2 was necessary for optimal expansion and sustained survival of the responding cells.[43] Interestingly, CD8$^+$ tg T cells engineered to be capable of prolonged IL-2R-mediated signaling showed a significant increase in expansion of CD8$^+$ cells in response to LCMV, followed by enhanced secondary responses upon re-exposure to antigen.[47]

Contrasting in part with these above-mentioned findings on IL-2, another study implicated an initial role for IL-15 in CD8$^+$ cell proliferation. This study examined polyclonal T-cell responses to alloantigens or superantigens in vivo and concluded that IL-15-driven initial cell division had to occur before IL-2 production; IL-2 synthesis and CD25 upregulation became evident towards the end of the T-cell expansion phase and IL-2 signaling during this stage decreased or even terminated T-cell proliferation via downregulation of the γ_c receptor.[48] For anti-viral responses, however, there is no evidence for downregulation of the γ_c receptor.[49] Moreover, administration of recombinant IL-2 (rIL-2) promotes the expansion of CD8$^+$ cells,[43,49] indicating that levels of γ_c receptors on the responding CD8$^+$ T cells are sufficient for IL-2 signaling. These data thus question the notion of a negative role for IL-2 at the end of the T-cell expansion phase because of γ_c downregulation. T-cell expansion is probably curtailed largely through loss of contact with antigen at the end of the primary response, thus leading to a decrease in the stimulus for IL-2 production. However, a decline in IL-2 production may also involve other factors. Here, it is noteworthy that the factor B-lymphocyte-induced maturation protein 1 (BLIMP1), a transcriptional repressor, has been suggested to regulate terminal differentiation of effector T cells by limiting IL-2 production and promoting activation-induced cell death (AICD).[50-52]

It is important to note the shortcomings associated with the use of IL-2$^{-/-}$ and IL-2R$^{-/-}$ mice. In particular, these mice develop hyperplasia of secondary lymphoid organs along with a multi-organ inflammatory disease, thus partly obscuring other immune responses.[53-55] Moreover, T cells developing in these animals do not receive any (or modified) IL-2 signals and develop in the absence of IL-2-dependent Tregs. In order to circumvent these problems, investigators have used a BM chimera approach where irradiated WT mice were reconstituted with a mixed population of T-cell-depleted BM from WT and CD25$^{-/-}$ mice, thus allowing a direct comparison of normal and CD25-deficient CD8$^+$ cell responses in a normal host. Subsequently, these mixed chimeras were infected with LCMV and virus-specific CD8$^+$ T cells from WT or CD25$^{-/-}$ origin and analyzed based on differential expression of congenic markers. One group of researchers observed only minimal differences in the responses of the two populations of CD8$^+$ T cells during primary expansion, regardless of whether polyclonal CD8$^+$ T cells or LCMV-specific TCR tg CD8$^+$ T cells were tested.[46] Conversely, also using LCMV infection (but another LCMV strain), others reported a 5-fold decrease in the primary expansion of CD25$^{-/-}$ polyclonal CD8$^+$ T cells as compared to their WT counterparts.[56] IL-2 signals through high-affinity IL-2Rs might thus be important for maximal expansion of virus-specific CD8$^+$ T cells. This finding is in line with the above data obtained using IL-2$^{-/-}$ mice.

Another approach for countering the severe pathology seen in IL-2$^{-/-}$ and IL-2R$^{-/-}$ mice is to limit IL-2 unresponsiveness selectively to peripheral T cells but not thymocytes. This has been done by generating CD122$^{-/-}$ tg mice that express CD122 under the CD2 promoter, thus leading to selective expression in thymocytes;[57] these mice do not display pathology and show normal CD8$^+$ cell development. With these mice, it was shown that signals through CD122 (the common receptor for IL-2 and IL-15) were dispensable for generating expansion and CTL activity of CD8$^+$ T cells following in vivo infection with recombinant vaccinia virus, injection of an agonistic anti-CD3 mAb or stimulation with superantigen.[57,58] Nevertheless, IFN-γ production was somewhat reduced, indicating that optimal stimulation required signaling through CD122.[57,58] Others obtained similar findings by preparing OT-I TCR tg CD8$^+$ T cells on a WT, CD122$^{-/-}$ or CD122$^{-/-}$ tg background and then transferring these cells to WT mice followed by administration of soluble ovalbumin; based on proliferation and generation of CTL activity in vivo, no significant difference was noted in antigen-specific CD8$^+$ T cells from these different backgrounds.[59] These results with CD8$^+$ cells from CD122$^{-/-}$ tg mice contrast with the above data obtained by others using IL-2$^{-/-}$ and CD25$^{-/-}$ mice.

The various approaches described above were aimed at determining the role of endogenous IL-2 at normal physiological levels. The results of exposing CD8$^+$ cells to exogenous IL-2 are considered below.

The effects of administering low-dose recombinant human (rh) IL-2 during expansion of virus-specific CD8$^+$ T cells was examined by giving mice two injections per day of 15,000 international units (IU) of rhIL-2 on days 0-8 after LCMV infection. Such IL-2 treatment resulted in similar LCMV-specific CD8$^+$ T-cell counts in IL-2-treated and control mice on day 8 after infection (though, surprisingly, numbers of virus-specific CD4$^+$ T cells at the peak of the response were markedly reduced in IL-2-treated mice compared to controls).[49] Moreover, IL-2 treatment did not affect LCMV clearance from the spleen and viral titers became undetectable 9 days after infection in both groups. These results suggest that provision of additional IL-2 during the first 8 days following LCMV infection does not influence primary expansion and effector function of virus-specific CD8$^+$ T cells (which contrasts with a negative influence on virus-specific CD4$^+$ cells). Interestingly, allospecific responses of 2C TCR tg CD8$^+$ T cells to BALB/c (H-2^d) splenocytes were found to be increased by about 40% following the administration of recombinant mouse (rm) IL-2 during the first 4 days of stimulation in vivo.[60] Moreover, for total T cells stimulated with superantigens, implantation of an IL-2-containing osmotic pump prolonged the expansion and survival of superantigen-reactive T cells as measured on day 6 after stimulation.[61] Thus, provision of exogenous IL-2 in these latter two settings might be beneficial because, under the conditions used, immune activation to alloantigens or superantigens was brief in the absence of IL-2 and therefore probably associated with much less proliferation than after virus infection. In the case of alloantigens, prolonged anti-host responses occur when T cells are transferred to irradiated

H-2-different mice, thus eliciting graft-versus-host disease (GVHD). For GVHD produced by purified $CD8^+$ cells, disease induction is much worse when the hosts are injected repeatedly with rIL-2 starting at 1 week posttransfer or when donor $CD8^+$ cells are co-injected with IL-2-producing $CD4^+$ cells.[62] Paradoxically, with a mixed population of T cells, administration of IL-2 from days 0-5 after T-cell transfer can protect against GVHD, perhaps by stimulating Tregs.[63]

Recently, it has been shown that naïve $CD8^+$ T cells can proliferate vigorously and differentiate into MP cells when exposed to high levels of IL-2 in vivo in the absence of antigen. Such proliferation occurs when naïve $CD8^+$ cells are transferred to $CD25^{-/-}$ or $CD122^{-/-}$ mice; not being able to utilize IL-2, these mice have high levels of IL-2 and also IL-15 in the case of $CD122^{-/-}$ mice.[64] Antigen-independent proliferation of naïve $CD8^+$ cells to IL-2 also occurs after administration of rIL-2 mixed with a particular anti-IL-2 monoclonal antibody (mAb).[45] This combination leads to the formation of highly stimulatory IL-2/anti-IL-2 mAb complexes, which under in vivo conditions are able to stimulate polyclonal or TCR tg naïve $CD8^+$ T cells to differentiate into effector cells able to produce IFN-γ, tumor necrosis factor-α and granzyme B as well as lysis of target cells.[64,65] Subsequently, these IL-2/anti-IL-2 mAb complex-stimulated $CD8^+$ cells differentiated into MP cells (for polyclonal cells) or central memory cells (in the case of TCR tg cells). For TCR tg cells, OT-I memory $CD8^+$ cells generated by activation with IL-2/anti-IL-2 mAb complexes in the absence of antigen conferred efficient protection against challenge with recombinant LM expressing ovalbumin.[65] Notably, despite being antigen independent, this form of IL-2-driven proliferation of naïve $CD8^+$ cells was found to be highly dependent on contact with self-peptide/MHC-I molecules, i.e., as for naïve $CD8^+$ cells undergoing IL-7-driven homeostatic expansion. It should be noted that IL-2/mAb complexes also considerably enhance antigen-driven responses. Thus, when IL-2/anti-IL-2 mAb complexes were injected plus specific antigen to stimulate influenza-specific TCR transgenic $CD8^+$ T cells in vivo, the complexes increased numbers of proliferating antigen-specific $CD8^+$ cells by 7-fold and conferred the cells with strong effector functions such as IFN-γ production and CTL activity.[66]

IL-2 and the Contraction Phase

As mentioned earlier, most effector cells are eliminated at the end of the primary response, thus leading to a sharp contraction in total numbers of antigen-reactive cells. When BM chimeras containing a mixture of WT and $CD25^{-/-}$ cells were infected with LCMV, the decline in virus-specific $CD8^+$ cell numbers during the contraction phase was similar for WT and $CD25^{-/-}$ cells.[46] Thus, IL-2 signals via the high-affinity IL-2R do not seem to influence contraction. By contrast, injection of IL-2 during the contraction phase does prevent elimination of the responding cells. Thus, treating mice twice daily with 15,000 IU rhIL-2 on days 8-15 after LCMV infection resulted in a marked reduction of T-cell apoptosis and increased survival, both for $CD8^+$ and $CD4^+$ cells.[49] This effect was seen in both lymphoid and nonlymphoid organs (such as liver and lungs), indicating that IL-2 had a direct effect in promoting cell survival rather than causing an alteration in cell migration. Following this short course of IL-2 therapy for 1 week, elevated numbers of LCMV-specific $CD8^+$ and $CD4^+$ cells persisted for about 6 months before reaching the levels found in control mice. This potentiating effect of IL-2 on $CD8^+$ T-cell counts was not a byproduct of the increase in $CD4^+$ numbers but reflected a direct action of IL-2 on $CD8^+$ cells: thus, the beneficial effect of IL-2 on $CD8^+$ cell numbers also applied in mice lacking $CD4^+$ T cells (either following depletion of $CD4^+$ T cells by antibody in normal mice or by using $CD4^{-/-}$ mice).[49] On a per-cell basis, the $CD8^+$ cells from IL-2-treated mice were roughly as efficient as their counterparts from control animals. Collectively, these experiments suggest that IL-2 therapy during the contraction phase of an anti-viral immune response results in increased immunity to virus that lasts for several months (Fig. 2).

Similar findings on the beneficial role of exogenous IL-2 during the contraction phase were observed for the response of OT-I TCR tg $CD8^+$ T cells to soluble ovalbumin or recombinant vesicular stomatitis virus expressing ovalbumin. Here, daily injections of IL-2 during the later stages of the primary response prevented elimination of the responding cells, although this effect lasted only for 1-2 weeks.[43,59]

Memory Cell Generation and Recall Responses

During expansion and proliferation of antigen-specific T cells a small subset of cells expresses high levels of IL-7Rα (also called CD127); these cells give rise to long-lived memory cells, suggesting a role for IL-7 signals in the generation of functional memory cells.[67]

Recently, it was shown that IL-2 signals received during priming are necessary for efficient secondary responses of $CD8^+$ T cell to viruses (Fig. 2).[46,56] Thus, for the above-mentioned mixed WT/$CD25^{-/-}$ BM chimeras, it was reported that LCMV-specific memory $CD8^+$ T cells of $CD25^{-/-}$ origin expanded only 4-fold upon secondary antigen challenge 150 days after primary infection, compared to 40-fold expansion of WT memory $CD8^+$ cells.[46] Another group showed a 30- to 40-fold reduction in secondary expansion of $CD25^{-/-}$ antigen-specific $CD8^+$ T cells, relative to WT cells, when measured 30-45 days after primary antigen challenge.[56] This defect was not due to impaired primary expansion or decreased homeostatic proliferation during the memory phase, although total numbers of virus-specific $CD25^{-/-}$ memory $CD8^+$ T cells were 2- to 5-fold reduced in comparison to their WT counterparts. Instead, the failure to mount an efficient secondary response following challenge was due to a lack of IL-2 signals during priming. Thus, injection of IL-2 in the form of IL-2/anti-IL-2 mAb complexes[45] during the primary response allowed virus-specific $CD25^{-/-}$ memory $CD8^+$ T cells to efficiently expand and survive following secondary antigen challenge;[46] injecting IL-2 during the secondary response, by contrast, was much less effective. Given that IL-2 acts through low-affinity IL-2Rs (CD122) on $CD25^{-/-}$ $CD8^+$ cells, it would be interesting to test whether enhanced levels of IL-15 could substitute for IL-2 in rescuing secondary responses of $CD25^{-/-}$ $CD8^+$ T cells. In this respect, the poor generation of memory by $CD25^{-/-}$ $CD8^+$ T cells also applies to normal $CD8^+$ cells primed in the absence of $CD4^+$ T cells.[68-70] As $CD4^+$ T cells are the main producers of IL-2 under steady-state conditions,[25] these findings consolidate the view that the optimal function of memory cells hinges on the precursors of these cells being exposed to IL-2 during initial priming. It should be mentioned that $CD8^+$ T cells themselves can produce significant amounts of IL-2 in viral infections, but presumably in amounts insufficient to replace the need for IL-2 "help" from $CD4^+$ cells.[71]

With regard to resting memory $CD8^+$ cells, it was mentioned earlier that these cells divide sporadically through contact with background levels of IL-15. As for MP cells, the turnover of antigen (LCMV)-specific memory $CD8^+$ (and $CD4^+$) cells is considerably enhanced following injection of exogenous IL-2 (or IL-15).[49] Since memory and MP cells have low expression levels of CD25, responsiveness of these cells to IL-2 (and IL-15) is mediated by low-affinity IL-2Rs.[45]

As for acute viral infection, IL-2 therapy can also lead to an increased frequency of virus-specific $CD8^+$ T cells during chronic viral infections. Thus, when mice infected with LCMV clone 13, which results in a chronic infection, were treated with low-dose rhIL-2 for 1 week, LCMV-specific $CD8^+$ cell counts increased by about 10-fold and serum viral titers decreased in 80% of the animals.[49] This efficient stimulation of $CD8^+$ cells by IL-2 is somewhat surprising as $CD8^+$ T cells found in chronic infections have only intermediate levels of CD122 and background levels of CD25 receptors,[72] which contrasts with the high levels of CD122 found on normal MP $CD8^+$ cells and memory $CD8^+$ T cells generated after acute LCMV infection.[19,73] Notably, the LCMV-specific $CD25^{-/-}$ $CD8^+$ T cells generated in mixed WT/$CD25^{-/-}$ BM chimeras declined rapidly during persistent viral infection.[56] Thus, for chronic viral infections, either exogenous or endogenous IL-2 signals seem to be beneficial or even crucial for the prolonged maintenance of the responding $CD8^+$ T cells; these signals have to be delivered through high-affinity IL-2Rs.[56]

Memory Maintenance and Homeostasis

As mentioned before, the few $CD8^+$ T cells surviving the contraction phase and becoming resting memory cells are kept alive and in occasional cell division through contact with IL-15 and IL-7; these cells do not need TCR interaction with self-peptide/MHC-I molecules.[13-15] Currently, there is minimal evidence that IL-2 has a direct role in memory maintenance or homeostasis, probably because the background levels of IL-2 are too low to affect resting cells. Nevertheless, it is notable that antigen-specific $CD8^+$ memory cells generated in the absence of $CD4^+$ T cells

gradually decrease during the memory phase.[69,70] This decrease might be due to a lack of $CD4^+$ cell-derived IL-2 signals. Interestingly, $CD8^+$ MP cells adoptively transferred to IL-$2^{-/-}$ recipients show a slightly reduced rate of homeostatic proliferation compared to WT recipients (O.B. and J.S., unpublished data). Hence, contact with endogenous IL-2 may play a significant, if minor role in memory $CD8^+$ cell homeostasis. As mentioned above, memory and MP $CD8^+$ cells are both strongly responsive to exogenous IL-2 signals.[45,49]

Indirect Roles of IL-2 in the Generation of Memory $CD8^+$ T Cells

Through its role in controlling the survival of $CD4^+$ Tregs, IL-2 plays a vital role in maintaining immune tolerance.[22,25,74,75] Tregs, which are typified by high expression of CD25 and forkhead box p3 (Foxp3) transcription factor,[34,76-78] may impair memory cell generation indirectly by several mechanisms, including inhibiting the intensity of the primary response, secreting inhibitory cytokines and consuming stimulatory cytokines, including IL-2.[79-83] The many inhibitory functions of Tregs on the immune response have been discussed elsewhere.[84-87]

Conclusion

In conclusion, IL-2 seems to have an important influence on $CD8^+$ cells at all stages of the immune response. Nevertheless, the evidence on this issue is still fragmentary and there are still substantial points of disagreement. For the primary response, most studies indicate that IL-2 is needed for optimal expansion and generation of effector functions. The discrepancies observed between individual studies may reflect the different systems used (pathogen vs. peptide stimulation) and/or differences in the precursor frequencies of antigen-specific $CD8^+$ T cells: thus, systems with high frequencies of responding antigen-specific $CD8^+$ cells may consume large amounts of stimulatory cytokines, thus accentuating a need for IL-2 for optimal expansion. Alternatively, as suggested by some studies,[43,48] IL-2 might not be necessary for initial division of $CD8^+$ cells but rather for the late stages of proliferation, thus accentuating the overall magnitude of the primary response and thereby increasing the total numbers of effector cells available for differentiation into early memory cell precursors.[35] Since 90-95% of antigen-specific T cells disappear by apoptosis during the contraction phase—perhaps largely because of loss of contact with growth factors—exposure to exogenous IL-2 during this stage can be highly beneficial in promoting cell survival and differentiation into early memory cells. Once these precursor cells upregulate CD122 and re-express IL-7Rα, memory cells become sensitive to the stimulatory effects of IL-15 and IL-7, IL-15 inducing intermittent cell division and IL-7 controlling survival.[9,10,19,45] At this stage, administration of exogenous IL-2 is able to boost the frequency of memory T cells, especially $CD8^+$ memory cells.[45,49] It is notable that IL-2 therapy is able to enhance proliferation of virus-specific $CD8^+$ T cells during the contraction phase but not during initial expansion. Why exogenous IL-2 is generally ineffective during the expansion phase is unclear. A likely possibility is that, during this stage, the stimulatory effects of IL-2 are countered by various negative influences, including enhanced sensitivity to BLIMP1-mediated AICD and suppression through the activation of Tregs.[51,52] Overall, IL-2 signals can be viewed as fine-tuning the immune response, boosting weak responses and inhibiting excessive responses, thus promoting an optimal response that eliminates the pathogen concerned while maintaining normal self-tolerance.

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Chapter 4

The Role of Inflammation in the Generation and Maintenance of Memory T Cells

Noah S. Butler and John T. Harty*

Abstract

Following infection or vaccination, antigen-specific T cells undergo enormous expansion in numbers and differentiate into effector cells that control infection and modulate other aspects of innate and adaptive immunity. The effector T-cell expansion phase is followed by an abrupt period of contraction, during which 90-95% of antigen-specific T cells are eliminated. The surviving pool of T cells subsequently differentiates into long-lived memory populations that can persist for the life of the host and mediate enhanced protective immunity following pathogen re-infection. The generation and maintenance of memory T-cell populations are influenced by a multitude of factors, including inflammatory cytokines that can act on T cells at various points during their differentiation. Herein, we discuss our current understanding of how inflammation shapes not only the quantity and quality of memory T cells, but also the rate at which functional memory T-cell populations develop.

Introduction

The generation and maintenance of efficacious, pathogen-specific memory T-cell responses is an important goal of vaccination. Moreover, following natural microbial infection, memory T cells afford heightened protective immunity against subsequent re-infection. Cell-mediated immunity is critically important for protecting the host against a multitude of pathogenic infections. $CD4^+$ T cells generally function to provide critical signals that augment both pathogen-specific $CD8^+$ T-cell and B-cell (antibody) responses. $CD8^+$ T cells are important in defense against viruses, intracellular bacteria and protozoan pathogens and are also potentially important in combating tumors. Upon reactivation, memory T cells exhibit an array of antimicrobial effector mechanisms and express molecules that mediate cytolysis of infected cells ($CD8^+$ T cells) or recruit and activate other immune cells through the elaboration of growth factors and/or inflammatory cytokines (both $CD8^+$ and $CD4^+$ T cells).[1] Thus, understanding factors that impact the development of memory T-cell populations following vaccination or natural infection remains an area of intense investigation.

Following infection or vaccination, dendritic cells (DC) acquire foreign antigens and undergo a program of maturation to become professional antigen-presenting cells (APC). This process culminates in the display of peptide/MHC complexes and costimulatory molecules on the cell surface, expression of inflammatory cytokines and migration from peripheral tissues to secondary lymphoid organs.[2,3] Naïve T cells continually traffic from the blood to secondary lymphoid organs where they survey antigens displayed by mature DC. Importantly, within hours following an infection- or

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vaccination-induced inflammatory response, lymphocyte exit from regional draining lymph nodes is blocked. This phenomenon is mediated by Type I IFN (IFN-α/β)-induced CD69 upregulation with subsequent internalization of sphingosine-1-phosphate receptor, a molecule critical for T (and B) cell egress from the lymph node.[4] Shutting down T-cell egress from the lymph node effectively increases the likelihood that DCs will encounter T cells expressing a relevant T-cell receptor (TCR). Following encounter with antigen-laden APCs, naïve T cells become activated and undergo a program of differentiation that can be grossly divided into four distinct phases (Fig. 1). In the first phase, stable interactions with APCs activate T cells via TCR signaling (signal 1) and APC-mediated

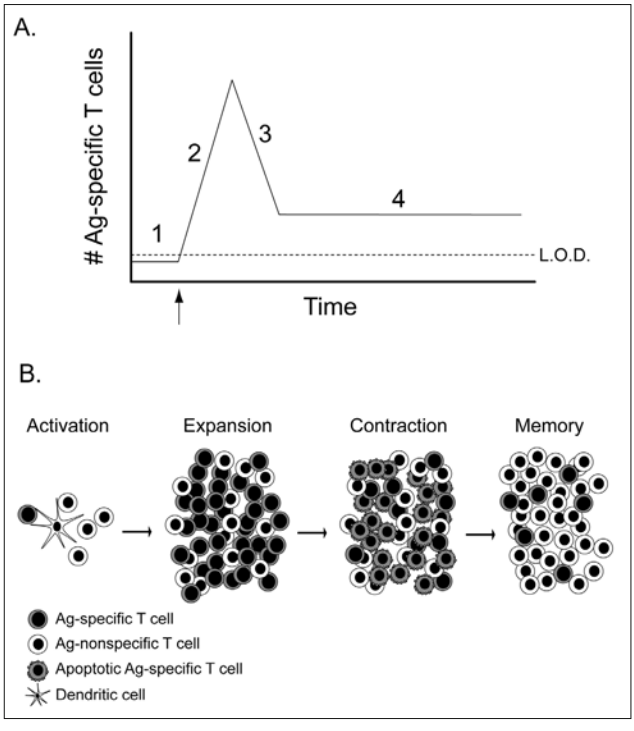

Figure 1. The magnitude and kinetics of antigen-specific T-cell responses following acute infection or vaccination. A) Schematic depiction showing the relative number of antigen-specific T cells as a function of time following acute infection or vaccination (arrowhead). The four phases of the T-cell response include; (1) priming and activation; (2) proliferative expansion and acquisition of effector cell function; (3) contraction; and (4) formation of stable memory populations. As described in the text, each of the four phases is influenced by proinflammatory cytokine signaling, acting either directly on T cells or indirectly through other cell types. Of note, prior to infection or vaccination, the number of antigen-specific T-cell precursors is below the limit of detection (L.O.D.), but the antigen-specific memory T-cell pool can be readily detected years following initiation of the response. B) Numbers of antigen-specific T cells following acute infection or vaccination change rapidly as the response transitions through the activation, contraction and memory phases. Following activation, antigen-specific T cells can undergo up to 13 rounds of cell division resulting in a >10,000-fold expansion in numbers. Seven to 10 days later, T-cell populations undergo abrupt contraction where in 90-95% of all antigen-specific T cells are eliminated. Over the next several months, the surviving antigen-specific T cells undergo slow homeostatic turnover, acquire phenotypic and functional properties of long-lived memory T cells and distribute to both secondary lymphoid and tertiary tissues. Importantly, the resultant pool of memory T cells is generally orders of magnitude larger than the naïve repertoir, which thus affords heightened immunity against re-infection.

costimulation (signal 2). In the second phase, activated T cells undergo robust expansion and acquire multiple effector functions. During this 5-8 day expansion period, pathogen-specific T-cell numbers increase by >10,000-fold,[5-7] and daughter cells express effector molecules and begin migrating from the lymphoid organs to the peripheral tissues to combat infection. In the third phase, the T-cell response undergoes contraction, wherein 90-95% of effector T cells that arose during the proliferative expansion are eliminated. Often the contraction phase occurs independent of pathogen/antigen clearance from the host, suggesting that a brief encounter with antigen during the initial activation phase is sufficient to initiate a full "program" of T-cell development.[8-10] Of note, T-cell contraction is incomplete and some pathogen-specific T cells survive and persist in nearly all tissues.[11] The persistence of these pathogen-specific cells comprises the fourth phase of the T-cell response, the formation and maintenance of stable memory T-cell populations. Importantly, the resultant memory populations generally can be maintained for the life of the animal and confer enhanced protective immunity against subsequent re-infection.

While it is well described that integration of signals 1 and 2 during the activation phase impacts the generation of efficacious T-cell responses, it is now becoming clear that additional signals, specifically inflammatory cytokines produced by APCs and other cells, act directly on T cells to influence T-cell survival and differentiation. In this chapter, we highlight both current and classic studies that have shaped our understanding of how and when inflammation/inflammatory cytokines influence the T-cell response. Importantly, the size of memory T-cell populations is directly related to T-cell expansion, degree of contraction and the sustained maintenance of memory T cells, thus the role of inflammation in shaping each of these phases of the T-cell response will be discussed. The major themes of this chapter are: (1) T-cell responses are largely programmed early following antigen encounter; (2) inflammation can influence each phase of the T-cell response; (3) inflammatory signals that act on T cells are largely pathogen- or adjuvant-specific; and (4) inflammation directly impacts the rate of memory T-cell generation.

The Role of Inflammation during T-Cell Priming and Expansion

T cells specific for any single pathogen-derived determinant constitute a small fraction of the naive repertoire, ranging from tens to hundreds of cells in the laboratory mouse.[12-14] Therefore, upon antigen encounter, naïve T cells must greatly expand in numbers to effectively eliminate pathogen-infected cells following infection. As noted above, the size of the memory T-cell pool is often proportional to the size of the effector T-cell pool that arises following infection or vaccination.[15] Thus, factors that influence the magnitude of T-cell expansion can directly impact the size of the stable memory T-cell pools that persist following contraction. As such, we will first discuss how inflammation influences T-cell priming and expansion

Activation of cells of the innate immune system (i.e., DC, macrophages, NK cells) occurs following pathogen infection or immunization in the presence of adjuvants. Innate immune cells respond to infectious agents via cell-surface and intracellular expression of Toll-like receptors (TLR) and other innate immune pattern-recognition receptors (PRRs). Ligation of these receptors in APCs by pathogen-derived molecules (which are also the primary consituents of vaccine adjuvants) can result in both upregulation of costimulatory molecule expression and secretion of a number of proinflammatory cytokines, including Type I interferons (IFN-α/β), Type II interferon (IFN-γ) and IL-12.[3,16,17] While these inflammatory cytokines further enhance the maturation of APCs,[17] they are also known to act directly on responding T cells (signal 3) and influence the development and survival of effector T-cell populations (Fig. 2).

For both CD4 and CD8 T cells, classic in vitro studies showed that the addition of IL-12 or IFN-α/β to T-cell cultures enhanced survival of proliferating T cells. These studies led to the hypothesis that signal 3 provided by inflammatory cytokines was important for optimal T-cell responses.[18-21] Direct proof of this concept came from a series of elegant in vivo studies demonstrating that pro-inflammatory cytokines can act directly on responding T cells to augment effector T-cell responses. These studies revealed significantly reduced expansion of adoptively transferred TCR transgenic (tg) T cells genetically deficient in receptors for IFN-α/β, IL-12 or

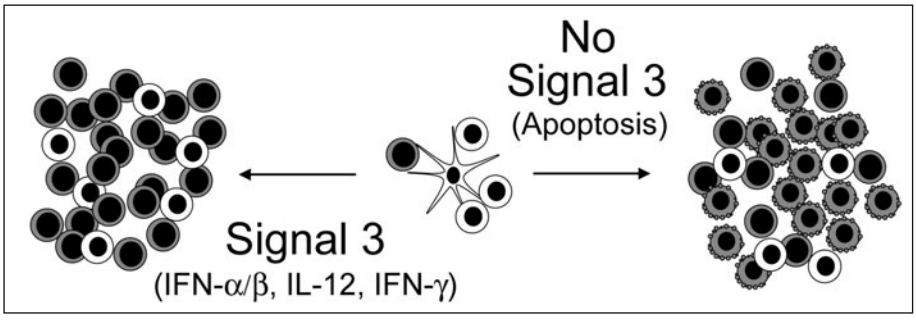

Figure 2. Signal 3 provided by proinflammatory cytokines is required early following antigen exposure to ensure survival of effector T-cell populations. Acute infection or vaccination in the presence of adjuvants results in the expression of proinflammatory cytokines such as IL-12, Type II IFN (IFN-γ) and Type I IFN (IFN-α/β) from both professional antigen presenting cells (dendritic cells) and other cells of the innate immune system (marcophages or natural killer cells). These cytokines, in turn, both augment further antigen presenting cell function and provide critical survival signals to responding T cells that prevent the immediate initiation of apoptotic cell death pathways.

IFN-γ, compared with wild-type TCR-tg T cells responding to lymphocytic choriomeningitis virus (LCMV) infection in the same wild-type recipient mice.[22-27] That fewer receptor-deficient T cells accumulate during the expansion phase demonstrates that T cells directly receive signals provided by inflammatory cytokines during their response to infection. Importantly, the absence of these inflammatory cytokine receptors did not alter the proliferative capacity of the T cells, rather the effect was directly related to decreased survival rates, resulting in reduced accumulation of receptor-deficient T cells during the expansion phase.[23-27] Interestingly and in stark contrast to results from studies utilizing adoptive transfer of Type I receptor-deficient T cells to WT recipient mice, LCMV infection of mice wholly deficient in Type I IFN signaling (IFNAR$^{-/-}$ mice) revealed T-cell expansion that was grossly normal.[28,29] While this discrepancy has not yet been formally resolved, it is possible that because IFNAR$^{-/-}$ mice fail to clear virus, T-cell proliferative capacity is altered, or the lack of Type I IFN responsiveness skews the balance of cytokine production toward use of alternative cytokines (e.g., IL-12 or IFN-γ) as signal 3. Regarding the in vivo effects of IL-12 signaling, a more recent study found, in line with results described above, that lack of IL-12 responsiveness in CD8^{+} T cells significantly reduces the expansion, survival and function of effector cells following *Listeria monocytogenes* infection.[30] As a composite, these data show that T-cell responsiveness to proinflammatory cytokines dramatically influences the differentiation of both virus- and bacteria-specific T-cell responses. Furthermore, the disparate results utilizing receptor-deficient T cells versus whole animal receptor-knockouts underscore complexity of in vivo proinflammatory cytokine production, the cytokine-responsiveness of T cells versus non T cells and the development of potent, pathogen-specific T-cell responses.

While the experiments outlined above revealed that IL-12, IFN-γ and IFN-α/β can each exert direct effects on T cells and influence the ensuing effector T-cell response, it is also important to point out results from several studies demonstrating that the nature of the pathogen can dictate the magnitude, kinetics and the profile of inflammatory cytokines expressed by infected cells and by cells of the innate immune system.[25,28,31,32] Differences in inflammatory cytokine profiles and kinetics of cytokine expression, in turn, affect when, how or whether various signal 3 cytokines influence T-cell expansion. As described above, TCR-tg T cells lacking the Type I IFN receptor fail to expand following LCMV infection,[23] however the same TCR-tg T cells that lack the IL-12 receptor or IFN-γ receptor exhibit only modest defects in expansion following LCMV infection. On the other hand, the lack of Type I IFN receptor expression has only minimal effects on T-cell expansion following *L. monocytogenes* or vaccinia virus infection.[25,33,34] Of note, IL-12 and IFN-γ

expression is rapidly induced following *L. monocytogenes* or vaccinia virus infection, whereas LCMV infection triggers delayed expression of these cytokines while rapidly inducing IFN-α/β expression.[25,28] Thus, each pathogen likely engenders different proinflammatory cytokine expression kinetics and cytokine profiles, which may in turn dictate which molecules function as signal 3 to ensure robust expansion and survival of effector T cells.

Although multiple proinflammatory cytokines can serve as signal 3 in vitro, the requirements for signal 3 cytokines that result in optimal pathogen-specific T-cell responses in vivo are less clear. Understanding the factors that determine which cytokines function as signal 3 following infection will require an in-depth analysis of the profile of proinflammatory cytokine production and the individual requirements of protective T-cell responses following infection by diverse pathogens. In addition to issues of timing and combinatorial complexity, another central question that remains relates to the molecular mechanism(s) by which inflammatory cytokines influence the expansion and survival of effector T cells. Although not completely understood, recent data suggest that proinflammatory signals (signal 3) serve to enhanced expression of prosurvival molecules such as B-cell lymphoma 3 (Bcl-3), which has been shown to be upregulated by IL-12 signaling.[35,36] Alternatively, data from other studies have shown that signal 3 can influence the balance of pro-apoptotic and anti-apoptotic molelcules of the Bcl-2 family in responding T cells.[37] Understanding the precise mechanisms by which signal 3 augments T-cell activation and effector T-cell survival will be an important goal for future studies. This information will likely be critical for selecting adjuvants to optimize both $CD4^+$ and $CD8^+$ T-cell responses to vaccination.

Inflammation and T-Cell Contraction

As described above, the expansion phase of the T-cell response is followed by an abrupt transition to a contraction phase, wherein 90-95% of pathogen-specific effector T cells are eliminated. Because the contraction phase is incomplete, the pool of pathogen-specific T cell that remain constitute the memory T-cell compartment (Fig. 1). Classically, the onset of the contraction phase coincides with pathogen/antigen clearance. This correlation was initially interpreted to mean that T cells somehow sensed antigen clearance and underwent a program of cell death that effectively generated 'space' for the immune system to respond to new infections. However, several experiments demonstrate that the onset and degree of T-cell contraction can occur independently of pathogen/antigen clearance.[8-10,38] In addition, T-cell responses that arise during many acute or chronic virus infections contract with similar kinetics.[8,39,40] Together, these results suggest that T-cell responses are 'programmed' following a relatively brief period of antigen stimulation after infection.

Several studies have revealed that at least two candidate signal 3 cytokines that regulate T-cell survival during the expansion phase (i.e., IFN-γ and IL-12) also influence the onset and degree of T-cell contraction. Following attenuated *L. monocytogenes* or LCMV infection of BALB/c mice genetically deficient in IFN-γ, pathogen-specific T cells expand normally, albeit expansion is somewhat reduced following LCMV infection.[41] However, despite relatively normal expansion in the absence of IFN-γ, T-cell responses in these mice fail to contract, an effect that is independent of pathogen clearance (described below). Additional studies have revealed similar phenomena in other mouse strains: T-cell contraction following LCMV infection is significantly reduced in C57BL/6 (B6) mice that lack either IFN-γ or its receptor.[41,42] While LCMV has been shown via RT-PCR to persist in these IFN-γ-deficient B6 mice,[43] more recent studies have shown that treatment of mice lacking IFN-γ or its receptor with antibiotics 4 days following *L. monocytogenes* infection (ruling out a role for antigen persistence) does not prevent abnormal contraction of $CD4^+$ or $CD8^+$ T cells.[44,45] In a series of complementary studies, it was shown that antibiotic treatment of WT mice prior to infection with *L. monocytogenes* significantly reduces levels of inflammatory cytokines, including IFN-γ and also significantly reduced T-cell contraction.[44] Importantly, reversing this effect via injection of a TLR9 agonist (CpG-containing DNA), to induce inflammation and IFN-γ secretion in the antibiotic-pretreated mice, restores contraction without altering the magnitude of T-cell expansion.[44] Moreover, memory cells that develop with or without contraction exhibit similar per cell protective

capacities following adoptive transfer into WT recipient mice,[44] suggesting that T-cell contraction is not required for the formation of functional memory T cells. How IFN-γ regulates contraction is still somewhat controversial. Although it was initially believed that IFN-γ primarily acted directly on T cells to regulate contraction, consistent with the behavior of IFN-γ receptor deficient T cells and the demonstration that T cells directly receive and respond to IFN-γ signals during the first 12 hours following *L. monocytogenes* infection,[46] more current studies indicate that IFN-γ can also act indirectly on other cell types to regulate T-cell contraction.[26,42] The relative importance of each pathway (direct or indirect effects of IFN-γ) in regulating T-cell contraction remains to be determined. As a composite, these data suggest that inflammatory signals provided by IFN-γ can directly and indirectly influence T-cell contraction and that progression through a contraction phase is not required for the generation of functional T-cell memory populations.

Regarding the role of IL-12, very recent work has determined that lack of IL-12 signaling in T cells results in an increase in numbers of *L. monocytogenes*-specific memory cells, suggesting that engagement of the IL-12 receptor on T cells not only plays a critical role in driving effector T-cell development (described above), but also influences memory CD8 T-cell development.[30] Other recent studies show that IL-12 signaling in $CD8^+$ T cells regulates the expression of the transcription factor T-bet.[47] The authors of this study conclude that graded expression of T-bet in recently activated effectors differentiates between short lived effector cells (SLEC, T-bet^{hi}), that do not survive the contraction phase, from memory precursor effector cells (MPEC, T-bet^{lo}) destined to become long-lived memory T cells. Of note, although these experiments reveal strong correlations between inflammation, levels of T-bet expression and the fate of T cells, evidence for a direct role for T-bet in regulating T-cell contraction is still lacking because T-bet-deficient tg T cells still undergo appreciable contraction.[47,48] Future studies are required to further define the precise role of inflammation in regulating the contraction of T-cell responses.

The link between proinflammatory cytokines and the pathways and molecular mechanisms responsible for cell death during T-cell contraction are not fully understood. In fact, much of the early research examining factors that influence T-cell contraction focused on pathways not intimately tied to inflammation. Indeed, early in vitro studies suggested that T-cell contraction is regulated via activation-induced cell death (AICD). However, follow-up studies demonstrated normal contraction of T cells lacking the death receptors (CD95 and TNFRI) implicated in classical AICD.[49,50] Attention subsequently turned to the regulation of (and competition for) common gamma-chain cytokines, particularly IL-2 and IL-7. Recently, several studies revealed an excellent correlation between the number of T cells expressing high levels of the IL-7 receptor α chain (CD127) at the peak of expansion and the number of Ag-specific $CD8^+$ T cells present following the contraction phase.[44,51,52] These data were interpreted to mean that IL-7 signaling directly promotes survival of T cells destined to become memory cells. However, more recent work shows that constitutive expression of CD127 on T cells is neither sufficient to prevent T-cell contraction following *L. monocytogenes* or LCMV infection[53,54] nor does high CD127 expression always correlate with diminished contraction under less inflammatory circumstances.[55,56] Of late, focus has again shifted toward examining the regulation, activation and balance of pro- and anti-apoptotic Bcl-2 family members that regulate mitochondrial integrity. For example, deletion of the Bcl-2 family member Bim prevents the massive deletion of T cells in vivo following exposure to staphylococcal enterotoxin B superantigen and significantly reduces T-cell contraction following betaherpesvirus challenge.[57,58] However, antigen-specific T-cell populations have also been shown to undergo rather normal contraction following LCMV infection of Bim-deficient mice[37,59] suggesting that additional pathways function to regulate apoptosis-driven cell death during T-cell contraction. Consistent with this, it was recently demonstrated that T cells lacking both CD95 and Bim contract less than T cells lacking Bim alone, particularly in lymph nodes.[60,61] Thus, additional studies are required to determine the relative contribution of death receptor- versus mitochondrial disruption-dependent mechanisms that determine the balance between effector T-cell survival and apoptotic death, as well as the influence that inflammatory cytokines have on initiating or potentiating these pathways.

In summary, contraction of T-cell responses appears to be programmed very early following T-cell priming and inflammatory cytokines, particularly IFN-γ and IL-12, appear to directly influence the timing and extent of T-cell contraction. Although there are no data to suggest that type I IFNs (IFN-α/β) regulate the onset or degree of contraction, at least following *L. monocytogenes* infection,[25,33,62] future studies will be required to determine whether additional proinflammatory cytokines (e.g., IL-6 or TNF-α) can alter programmed T-cell contraction. It will also be important to determine whether additional proinflammatory cytokines act directly on pathogen-specific T-cell populations or whether their effects are mediated indirectly through the activation of and subsequent cytokine production by other immune cell types. Lastly, it will be of interest to determine the molecular mechanisms and pathways by which inflammation regulates T-cell death and contraction of the T-cell response. This information will significantly broaden our understanding of basic T-cell biology and may ultimately prove useful in tailoring therapeutic or interventional strategies designed to either limit or enhance T-cell differentiation and survival.

Inflammation Regulates the Rate of Memory T-Cell Generation

There is some controversy in the field of memory T-cell biology regarding when memory T-cell populations become established following infection or vaccination and definitions of memory often appear specific to individual model systems. However, even though no single phenotypic marker (cell surface molecule or cytokine expression pattern) accurately defines a memory T cell, the use of multi-parameter flow cytometry in combination with methods to detect antigen-specific T cells (e.g., intracellular cytokine staining (ICS)[63] or MHC tetramer staining[64]) has enabled investigators to characterize the changing phenotype of T cells as they progress from naïve to effector to memory T cells (Table 1). When antigen-specific T-cell populations are characterized at various times following the contraction phase it becomes immediately clear that memory T cells are not a uniform population, but that extensive heterogeneity exists at the population level. Despite this heterogeneity, effective memory T-cell populations can be classified as exhibiting the following properties: (1) they persist at numbers higher than the naive repertoire; (2) they rapidly respond to re-infection utilizing multiple effector functions (e.g., cytolysis and cytokine production); (3) they provide enhanced protection against re-infection; and (4) they expand vigorously following antigen re-encounter (the formation of secondary memory T cells), resulting in increased numbers and increased functionality as compared to primary memory T cells. Importantly, experimental

Table 1. Changes in cell surface marker and effector molecule expression in populations of T cells differentiating from naïve to effector to memory

Marker	Naïve	Effector	Early Memory	Late Memory
CD62L	+++	–	+	+++
CD127	+++	–/+	+	+++
CCR7	+++	–/+	+	+++
CD43	+	+++	++	+
CD44	+	+++	+++	+++
KLRG-1	–	+++	++	+/-
IL-2	–	+/–	+	++
TNF-α	–	+/–	+	+
Granzyme B	–	+++	–	–
IFN-γ	–	+++	+++	+++

settings that shift the kinetics of memory T-cell development (as assessed by both phenotypic and functional properties) have been instrumental in shaping our understanding of how inflammation regulates memory T-cell development (Fig. 3).

An acute infection in otherwise normal host results in T-cell responses that undergo explosive expansion, rapid contraction and are characterized by a relatively slow conversion from effector to memory cell phenotype and function (Fig. 3A). However, the rate at which T cells acquire characteristics of functional memory cells is not fixed and is often pathogen-dependent. For example, $CD8^+$ T-cell phenotypic differentiation to memory following *L. monocytogenes* infection occurs relatively rapidly (1-2 months) as compared to LCMV infection (greater than 6 months),[40,65] even when examining T cells of identical antigen specificity. While the rate of conversion to memory in each of the above scenarios is perhaps related to differences in target cell tropism, pathogen replication or strength of TCR signaling,[66] it is also likely that the profile of inflammatory cytokines expressed following virus versus bacterial infection influences T-cell progression to memory. Indeed, experiments utilizing *L. monocytogenes* have made it very clear that inflammation significantly impacts the rate of memory T-cell differentiation. For example, in mice that received antibiotics 48 hours following attenuated *L. monocytogenes* infection (to rapidly clear infection and reduce inflammation), antigen-specific CD8 T cells acquire memory T-cell phenotype ($CD62L^{hi}$, KLRG-1^{low}, $CD127^{hi}$, $CD27^{hi}$) by day 14 post-infection, in the absence of alterations to the magnitude and peak of expansion and onset of contraction[65] (Fig. 3B). Furthermore, T-cell responses generated in this manner could be boosted two weeks following infection, thus establishing the accelerated formation of functional memory CD8 T cells. Importantly, these effects were reversed by administration of CpG DNA to induce inflammation.[65] In companion studies, it was shown that treatment of mice with antibiotics prior to *L. monocytogenes* infection results in a >99% decrease in bacterial load at 24 hr post-infection infection, diminishes serum proinflammatory cytokine levels and markedly impairs $CD8^+$ T-cell expansion. However, despite reduced expansion, $CD8^+$ T cells primed in antibiotic-pretreated mice develop phenotypic (KLRG-1^{low}, $CD43^{low}$, $CD62L^{hi}$, $CD127^{hi}$, $CD27^{hi}$) and functional (IL-2-producing and responsive to boosting) characteristics of long-term memory $CD8^+$ T cells within 6 days after immunization[65] (Fig. 3C). Together, these data show that the duration of infection-induced inflammation (rather than simply the duration of infection) regulates the differentiation to memory and that in the absence of overt, systemic inflammation the generation of functional memory T cells is accelerated.

In addition to manipulating the rate of memory cell development in the *L. monocytogenes* model, our lab has demonstrated that vaccination of mice with peptide-coated DCs also results in the accelerated formation of memory CD8 T-cell populations[55] (Fig. 3D). In this model, inoculation of mice with mature, peptide-coated DCs results in the rapid differentiation of $CD8^+$ T cells exhibiting functional and phenotypic properties of memory cells. Indeed, as early as 4-6 days following peptide-DC vaccination, the majority of Ag-specific $CD8^+$ T cells exhibit characteristics ($CD62L^{hi}$, $CD127^{hi}$, $CD27^{hi}$, KLRG-1^{low}, IL-2 production) of long-term memory T cells. Moreover, these memory-like cells are able to proliferate in response to booster immunization as soon as 4 days following DC priming. Co-infection of mice with *L. monocytogenes* or co-administration of CpG DNA to induce inflammation prevents the accelerated memory $CD8^+$ T-cell transition after peptide-DC vaccination, without changing the magnitude of Ag-specific $CD8^+$ T-cell expansion. Additional studies utilizing adoptive transfer of TCR tg T cells demonstrated that duration of functional antigen presentation by injected DCs lasted only approximately 2 days. Moreover, CpG DNA could be administered up to 3 days following DC vaccination and still prevent the accelerated development of memory CD8 T cells, suggesting that inflammation is not acting through the injected DCs. Lastly, when IFN-γ receptor-deficient mice are DC-vaccinated, co-administration of CpG DNA does not prevent rapid memory $CD8^+$ T-cell development.[55] These data suggest that IFN-γ-mediated inflammation (but not necessarily IFN-γ itself) received by responding $CD8^+$ T cells controls the rate of memory $CD8^+$ T-cell differentiation.

In addition to modulating the rate of memory differentiation, inflammatory cytokines have recently been implicated in the rapid acquisition of effector functions by memory T cells. Rapid

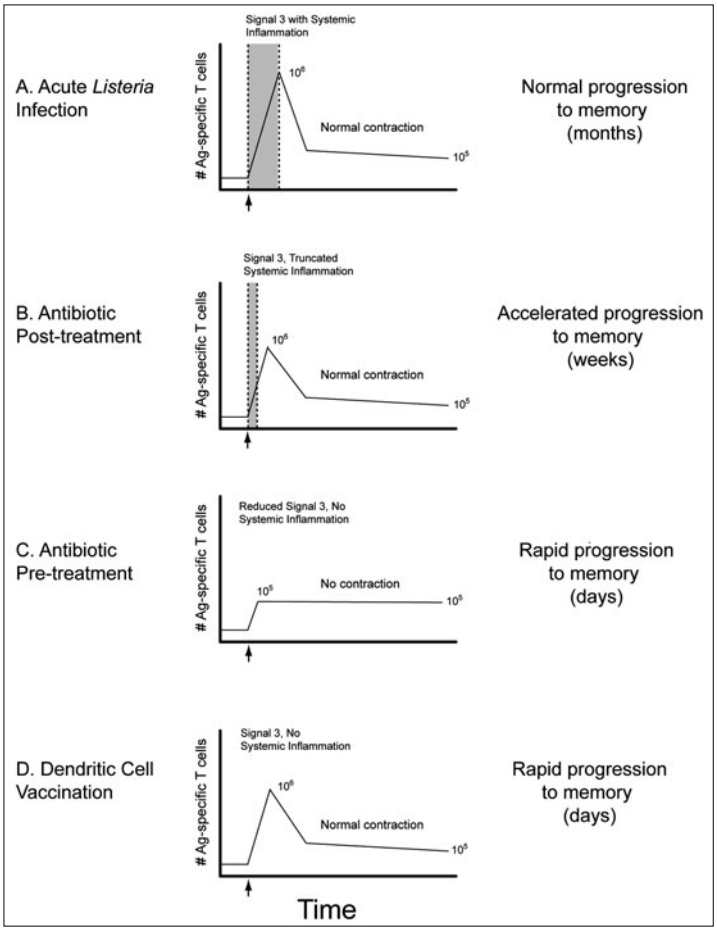

Figure 3. Inflammation regulates the rate memory T-cell differentiation. A) Following acute infection or vaccination (arrowhead) of an otherwise unmanipulated host, antigen-specific T cells undergo expansion, contraction and subsequently generate a stable population that acquires phenotypic and functional properties of memory T cells over the course of several months. In this scenario, mature DCs provide ample MHC/peptide (signal 1), costimulation (signal 2) and inflammatory cytokines (signal 3) to antigen-specific T cells resulting maximal proliferative expansion of effector T-cell populations. Infection-associated systemic inflammation (shaded to indicate duration) modulates the onset and degree of contraction and the protracted rate of memory T-cell differentiation. B) Truncating the systemic inflammatory response following *L. monocytogenes* infection by the post-infection administration of antibiotics does not change signal 1, 2, or 3, or the kinetics of the T-cell response (i.e., expansion, contraction and memory T-cell numbers), but does accelerate the rate of memory T-cell differentiation. C) Administration of antibiotics prior to infection with *L. monocytogenes* attenuates bacterial replication, significantly diminishes signals 1, 2 and 3 and prevents infection-associated systemic inflammation. In this scenario, the T-cell response exhibits reduced expansion, fails to contract and antigen-specific T cells rapidly (within days) acquire phenotypic and functional properties of memory T cells. D) Vaccination of mice with mature, peptide-coated dendritic cells (DCs) reveals a scenario in which antigen presenting cells provide adequate signal 1, 2 and 3 in the absence of overt, systemic inflammation. $CD8^+$ T-cell populations primed in this manner exhibit normal expansion and contraction. However, antigen-specific cells rapidly (within days) acquire phenotypic and functional properties of memory CD8 T-cell populations.

proliferation, at least for memory CD8+ T cells, appears to involve a signaling loop between stable DC-memory T-cell conjugates.[67] In this scenario, memory (but not naïve) T cells can rapidly express IFN-γ upon antigen/APC re-encounter. T-cell-derived IFN-γ potentiates the production of IL-18 from DCs, which in turn drives the rapid proliferation of memory T cells. The molecular basis for rapid proliferation and cytokine production by memory cells appears to be regulated by changing patterns of histone acetylation.[68] Thus, rapid proliferation and effector molecule induction correlate with epigentic changes that have occurred during memory T-cell differentiation.

While these experiments have made clear the impact inflammation has on the conversion of naïve T cells to functional memory, important questions still remain. For example, it is still not specifically known which proinflammatory cytokines elicited by *L. monocytogenes* infection or CpG DNA treatment reverses the accelerated progression to memory. The adoptive transfer of cytokine receptor-deficient CD8+ T cells to WT mice prior to peptide-DC/CpG DNA co-administration may reveal which molecules can provide the signal that balances effector versus memory T-cell development. In addition, the "window of opportunity" for proinflammatory cytokine signaling in T cells has not been investigated. For instance, can inflammatory signals act on T cells prior to antigen exposure and prevent the accelerated transition to memory in the DC-vaccination model? Lastly, very little is known about whether these molecules act directly on the T cell, or via signaling in other cell types. Future studies designed to address these questions should significantly broaden our understanding of how and when T cells receive inflammatory signals that influence the formation of functional, long-lived memory populations.

Collectively, these data reveal the interesting dichotomy that exists between the 'positive' influence of proinflammatory cytokines in promoting effector T-cell responses and the 'negative' influence of proinflammatory cytokines in limiting the development of memory T-cell populations. Thus, some proinflammatory cytokines (e.g., IL-12 and IFN-γ) are double-edged swords, at the same time enhancing and limiting various aspects of T-cell differentiation. Clearly, a balance has evolved between generating efficacious T-cell responses that can immediately participate in combating infection and the generation of memory responses that will enhance protective immunity to re-infection.

Memory T-Cell Maintenance

It has long been appreciated that certain memory T-cell populations are antigen-independent and several elegant studies demonstrated that memory T cells can persist following adoptive transfer into naïve mice[69] (lacking cognant antigen) or MHC-deficient mice[70,71] (lacking both antigen and TCR ligand). As descibed above, populations of long-lived memory T cells persist and maintain steady numbers for the life of the host. In circumstances where a pathogen or antigen is rapidly cleared (i.e., acute infection or vaccination), the maintenance of memory T-cell populations depends on direct signals provided by IL-7 and IL-15, which promote survival and slow homeostatic proliferation of memory T cells, respectively.[72-77] Because memory T-cell numbers are stably maintained, proliferation must be accompanied by an equal rate of memory T-cell death.

In contrast to scenarios of acute infection or vaccination, peristent infection and chronic antigen display often, but not always,[78] causes either deletion or significant alterations in both the phenotype and function of long-term memory T-cell populations. For example, following LCMV clone 13 infection (which establishes persistent infections in laboratory mice), virus-specific T-cell populations undergo functional "exhaustion" whereby they lose effector function and fail to mediate protective immunity.[79,80] Further, exhausted memory cells appear not to undergo IL-7- and IL-15-dependent homeostatic proliferation, as evidenced by their downregulation of IL-7 and IL-15 receptors (CD127 and CD122).[81-83] Instead, their maintenance requires continued antigen-driven proliferation and exhausted cells rapidly disappear following adoptive transfer to naïve mice.[83] In addition to down-regulation of CD127 and CD122, the molecular basis for these

phenomena appear to also involve the dysregulated expression of programmed cell death 1 (PD-1) receptor on exhausted memory T cells. Indeed, blocking PD-1 signaling in vivo enahances virus clearance and the function of exhausted memory CD8 T cells.[84]

These studies clearly indicate that the maintenance of memory cells is a function of growth factor-driven survival and proliferation and that chronic antigen/infection often drives the exhaustion of these populations. However, much less is known about the potential links between proinflammatory cytokine expression and memory T-cell maintenance and function. While several reports demonstrate a correlation between chronic inflammation and dysregulated memory T-cell maintenance or function,[85-87] only recently have investigators begun to directly address the connection. For example, a recent report by Dudani et al,[88] demonstrates decay of preexisting memory CD8$^+$ T-cell populations following heterologous bacterial challenge. The loss of these memory cells was dependent on IFN-γ signaling, as memory cells were able to persist following heterologous infection of mice lacking the IFN-γ receptor. While these data indicate a role for IFN-γ, it still remains to be determined whether IFN-γ signaling acts directly on the preexisting memory CD8 T cells. Clearly much more work is required to determine whether acute or chronic inflammatory settings directly impact the persistence of long-lived memory T-cell populations. Specifically, it will be of interest to determine which individual cytokines can (for better or worse) influence the maintenance of memory T cells and whether memory T cells directly receive simulation by inflammatory cytokines.

Conclusion

In this chapter we have highlighted experimental studies that have begun to reveal how and when pro-inflammatory cytokines influence the generation and maintenance of memory T-cell populations. Following infection or vaccination, professional antigen-presenting cells, through differential TLR activation, express unique patterns of proinflammatory cytokines. The magnitude, kinetics and specific profile of the inflammatory response can in turn differentially impact T-cell differentiation shortly following antigen encounter. As discussed above, proinflammatory cytokines also directly affect proliferative expansion, programmed contraction and the rate of memory T-cell differentiation. While several proinflammatory cytokines can regulate multiple phases of T-cell differentiation (e.g., IFN-γ and IL-12), others appear only to modulate discreet phases (e.g., IFN-α/β augmented effector T-cell survival). Collectively, these results indicate that different pathogens and adjuvants have the potential to uniquely shape the generation of long-lived, efficacious memory T-cell populations. In this regard, future studies aimed at identifying the precise signals that influence the development of T-cell responses will be of significant interest. Understanding the molecular basis for how proinflammatory cytokines (and the timing of their delivery) shapes the generation and maintenance of memory T cells populations will have important implications for vaccine development.

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Chapter 5

The Role of OX40 (CD134) in T-Cell Memory Generation

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Abstract

Memory T-cell generation is limited by activation-induced cell death during the effector T-cell stage. Cell surface proteins are known to transmit signals that either accentuate or limit T-cell death after activation. This chapter will focus on the TNF-receptor family member OX40, which is expressed on effector T cells and when engaged greatly enhances survival of T cells leading to increased memory T-cell generation. Targeting OX40 in vivo can alter the fate of T-cell survival. Enhancing OX40 signaling during Ag priming through agonists increases memory T-cell development, while blocking OX40 signaling decreases the memory T-cell pool. These two opposing outcomes provide therapeutic tools for blocking inflammation in autoimmune conditions and enhancing immunity in hosts harboring cancer or chronic pathogens. OX40 agonists and antagonists are in the first stages of human clinical trials and their therapeutic potential will soon be realized.

Introduction

The generation of functional immunologic memory via long-lived T- and B-cell responses is paramount to protective immunity against recurrent pathogen infections and is the goal of current vaccine strategies.[1] The coordination of long-lived CD4, CD8 and B-cell responses is a hallmark of the adaptive arm of immunity and is an irreplaceable part of protective immunity. The quality of the long-term adaptive immunity is directly related to the amount of Ag-specific memory T and B cells that are generated following an initial Ag challenge.[1] Hence, understanding the mechanisms that regulate the generation and maintenance of immune cells could lead to improved vaccine strategies and also help hosts harboring chronic pathogens or cancer tip the balance towards immune clearance and host survival. In particular, this chapter will explore the contributions of the TNF-receptor family member, OX40, to T-cell memory generation and how to exploit OX40-specific pathways for clinical benefit in patients with autoimmunity, cancer and chronic pathogens.

There has been a number of T-cell surface molecules linked to the biologic function of memory T-cell generation and function. The list includes several TNF-receptors, 4-1BB, FAS, LT-βR, CD27 and OX40 as well as other proteins such as CD28, ICOS, ICAM and PD-1. While all of these cell surface proteins have been linked to the function of memory T cells, it is clear that OX40 plays a seminal role in the generation of both CD4 and CD8 T-cell memory.[2] T-cell responses to cognate Ag are characterized by an early expansion phase, a contraction phase, followed by the generation and persistence of long-term memory cells.[1] The generation of large

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numbers of long-term memory T cells is limited by activation-induced cell death (apoptosis) during the contraction phase. Recently, it has been shown that engagement of OX40 during Ag priming in vivo diminishes T-cell activation-induced cell death leading to increased numbers of long-lived antigen-specific CD4 and CD8 T cells.[3-7] This chapter will review the events that lead to OX40 enhanced T-cell memory generation through natural OX40 ligand engagement (endogenous OX40 activation) or via exogenous administration of OX40 agonists (OX40 ligand:Ig or anti-OX40) in vivo.

Background

OX40 has a unique pattern of expression; it is for the most part restricted to lymphoid tissue[8] and mainly expressed on activated CD4 and CD8 T cells.[9] More recently, it has been shown that OX40 is also constitutively expressed on mouse T regulatory cells.[10] OX40 expression on recently activated naïve CD4 T cells peaks within 24-48 hr after TCR engagement by peptide Ag in the context of MHC class II and returns to baseline levels 120 hr later.[11] Effector CD4 T cells upregulate OX40 expression more rapidly than naïve T cells within 4 hr after Ag stimulation.[11] The transient expression of OX40 on activated effector cells is observed both in vitro and in vivo.[11,12] OX40 expression on naïve CD8 T cells starts 24 hr after Ag stimulation, peaks at 48-72 hr and declines thereafter.[5] OX40$^+$ T cells are found preferentially at sites of inflammation and not normally in the peripheral blood. In animal models for both autoimmunity and cancer OX40$^+$ T cells found within the site of disease are enriched for the recently stimulated auto- or tumor Ag-specific T cells.[13-15] Therefore, OX40 represents a convenient target by which the function of Ag-specific T-cell responses can be modulated in various disease models, even without prior knowledge of the specific Ag(s) involved.[16] In essence, manipulation of OX40$^+$ T cells in vivo targets the ongoing "endogenous" immune responses, but does not affect the remainder of the peripheral T-cell repertoire. OX40$^+$ T cells have been detected at the inflammatory site in several human autoimmune diseases and in the following human cancers: melanoma, breast, colon, head and neck and more recently prostate cancer, bladder cancer, lung cancer and sarcoma[16-18] and data not shown. Therefore, manipulation of OX40$^+$ T cells in patients with a variety of diseases could have a wide range of clinical benefits.

The original description of the OX40 monoclonal antibody (Ab) showed that this antibody bound activated CD4 T cells and augmented their proliferation during the later stages of in vitro stimulation.[9] When the biologic effects of anti-OX40 were originally described, the field of costimulation was in its infancy. CD28 was the first costimulatory molecule described on T cells; it was shown to augment T-cell stimulation when administered in combination with TCR signaling.[19-21] The CD28 interaction with its ligands (CD80/86) is essential to achieve optimal activation of naïve T cells; if a signal is delivered through the TCR receptor in the absence of CD28 ligation, the T cell becomes anergic or dies prior to becoming small resting T cells.[21] CTLA-4 is expressed after TCR engagement and competes with CD28 for binding CD80/86 and when engaged provides a negative signal that puts the brakes on T-cell proliferation. OX40 was originally shown to have costimulatory activity on an Ag-specific CD4$^+$ T-cell line in vitro of similar potency to that of CD28.[22] While interaction of B7/CD28 is required for the optimal stimulation of naïve T cells,[23] OX40-specific costimulation appears to be most important for the stimulation of effector T cells.[11,24] Both CD28 and OX40 appear to play important but distinct costimulatory roles in the development of Ag-activated peripheral T cells and both signals are required for the optimal generation of memory CD4$^+$ T cells.[7] The remainder of this book chapter will focus on the mechanistic details of how OX40 functions to increase effector T-cell survival/function ultimately leading to increased generation of a functional memory response. Ultimately, manipulation of OX40 signaling could beneficially alter the course of several diseases and this concept will be further dissected within this book chapter.

Role of OX40/OX40L Interaction in Memory T-Cell Generation and Function

OX40 expression is upregulated upon TCR engagement even in the absence of a strong innate immune adjuvant, however expression of the OX40 ligand is somewhat limited, especially in the absence of innate immune signaling.[25,26] Hence, the biologic role of endogenous OX40/OX40L interaction has been easier to ascertain in models where a proinflammatory event occurs causing innate immune activation (e.g., viral challenge, EAE, or asthma). It is now evident that the extent of OX40L expression regulates the magnitude of OX40 signaling within activated T cells.[27] The OX40 ligand (OX40L) is a Type II transmembrane protein (TNF-family member) that was first identified in mice and shown to have ~67% homology to the human protein gp34.[28] Subsequently, studies revealed that the gp34 protein could bind to human OX40, demonstrating that gp34 is the human homologue of OX40L and to date this is the only known ligand that binds OX40.[8,29] OX40L is expressed on activated APCs including B-cells, macrophages, DCs, NK cells, Langerhans cells, human airway smooth muscle (ASM) cells, CD4+ CD3- accessory cells and activated vascular endothelial cells and appears to be induced in a CD40-dependent manner.[25,30-39] The OX40L is also expressed by T cells and confer T:T-cell interaction that may also be important for OX40 signaling.[40]

There were two sentinel studies describing OX40/OX40L knockout mice, which suggested that memory T-cell generation was impaired upon targeted disruption.[41,42] The initial study challenged OX40 knockout mice with LCMV and influenza viruses. While these investigators observed no differences in Ag-specific CD8 T-cell memory and Ab responses, there was a significant decrease in viral-specific CD4 T-cell memory. In particular, there was a significant reduction in lung infiltration of $CD4^+$ T cells in virally infected OX40 knockout mice compared to controls. The second study described defective recall responses in OX40L knockout mice. This group immunized WT and OX40L ko mice with various Ag(s) and found defective proliferation and cytokine production within the CD4 T-cell compartment nine days following immunization. They also found defective Th1 and Th2 responses following restimulation in vitro, suggesting that the OX40 signaling can stimulate both Type 1 and 2 responses.[41] This study also revealed a decline in Ag-specific Abs (all isotypes) in KLH immunized OX40L ko mice and a slight decline in CD8 T-cell cytotoxicity.[41] Soon to follow was a manuscript detailing a marked decrease in CD4 T-cell memory following immunization of OX40 ko mice.[43] This study immunized mice with KLH either in alum (i.p.) or CFA delivered s.c. Similar to the previous study they found a marked decrease in Ag-specific cytokine production seven days after priming (effector T-cell stage). This study also investigated long-term survival of Ag-specific CD4 T cells and found a profound decrease in memory T-cell frequency and Ag-specific cytokine production in the OX40 ko mice. The frequency of long-term Ag-specific memory T cells decreased 11-fold and 23-fold in the CFA and Alum immunized OX40 ko hosts, respectively. This group further reported that an OX40-specific defect that led to decreased survival of Ag-stimulated T cells was in part due to decreased expression of the anti-apoptotic proteins, Bcl-2 and Bcl-xl.[7]

CD4 T-cell memory responses within the lung are a critical component for the induction of asthma in mouse models.[44] OX40 expression by T cells appears to be an essential component of T-cell-mediated lung inflammation in asthma, as OX40 ko mice develop a tempered form of the disease.[44] In addition, this group found that Ag-specific long-term memory T cells (120 days post-Ag priming) were also dependent on OX40 signaling to induce asthma.[45] This data suggest that OX40 signaling of long-term memory T cells is critical for their effector function and blocking OX40 signaling in vivo may have clinical implications for individuals with asthma.

Recent literature has focused on two phenotypes of memory T cells that have separate functional properties, effector and central memory.[46-48] Effector memory T cells reside in both lymphoid and nonlymphoid tissue, where they elicit immediate function by producing cytokines and/or being cytotoxic with little clonal expansion upon reencountering Ag.[46-48] In contrast,

central memory T cells are mainly located in the secondary lymphoid tissues, where they mediate long-lasting protection through clonal expansion.[46-48] A recent study showed a dramatic decrease in the effector memory population after Ag stimulation of OX40 deficient CD4 T cells compared to their WT counterparts.[49] There was no difference in these two populations three days after activation (as defined by CD62L and CD44), however as the cells became long-term memory T cells there was a dramatic loss in the effector memory population. The data also suggested that an OX40-specific signal generated early in the immune response is important to maintain these long-term effector memory CD4 T cells.[49]

The importance of CD8 memory T-cell generation in the context of OX40 signaling has been studied in both viral and tumor model systems.[50] Initial studies used OX40 deficient T-cell receptor transgenic T cells (OT1) and compared them to WT cells upon adoptive transfer in tumor-bearing mice (EG7). These investigators found that the survival of the OX40 ko OT1 T cells was diminished compared to WT T-cell transfers into tumor-bearing mice and this also correlated with diminished anti-tumor activity. Transfecting the anti-apoptotic gene, Bcl-xl, into the OX40 ko CD8 T cells enhanced survival of these cells and increased their efficacy against an ova-expressing tumor. Another study investigated influenza-specific CD8 T-cell priming and memory T-cell expansion in the absence of OX40 signaling (OX40 ligand ko mice).[51] They found that primary expansion and memory CD8 T-cell survival was not affected in the OX40 ligand ko mice, however upon viral rechallenge the influenza-specific T cells within the OX40 ligand ko hosts showed defective recall responses. Subsequent experiments showed that the defect in secondary expansion of viral-specific CD8 T cells was conferred to the cells during the initial priming phase.[51]

Providing an Exogenous OX40 Signal (OX40 Agonists) to Enhance Memory T-Cell Generation

The control point for OX40-dependent stimulation of T cells during an immune response appears to be at the level of OX40L expression. OX40 is expressed on all CD4 and CD8 T cells after TCR engagement. The expression of OX40L, however, is more tightly regulated. When T-cell activation via TCR engagement with peptide/MHC occurs in the absence of a strong adjuvant, the local expression of OX40L is minimal. Therefore, in the absence of adjuvant, the Ag-stimulated T cells express OX40, but because OX40L expression on APC is limiting the majority of $OX40^+$ T cells will never encounter/engage their natural ligand. This may lead to apoptosis and limit the generation of memory T cells as depicted in Figure 1. Evidence in support of this theory derives from two transgenic mouse models in which mice over express the OX40L.[27,39] In both models, the investigators noticed a large increase in the proportion of T cells in the lymphoid compartments as the mice aged. The OX40L transgenic mice also showed a dramatic increase in memory T-cell generation and recall responses following immunization.[27] Hence, one might predict that the addition of an exogenously delivered OX40 agonist (anti-OX40 or OX40L:Ig) during an ongoing immune response may increase the numbers of memory T cells generated.

The exogenous OX40 agonist hypothesis was initially tested in superAg-stimulated mice, which induces rapid in vivo T-cell expansion followed by deletion.[3] An OX40 agonist was administered at the same time as superAg to test whether this strategy might save T cells from clonal deletion. OX40 agonist administration was able to slightly increase T-cell survival in superAg (SEB) treated mice, similar to that observed with the TLR agonist, LPS.[3] However, combining an OX40 agonist with LPS in SEB treated mice showed dramatic synergy not only enhancing CD4 T-cell survival (greater than 2 logs), but also increasing the proliferative phase of T-cell expansion. This same dual adjuvant combination (anti-OX40/LPS) also provided increased survival of SEB specific CD8 T cells, although not as dramatic as the CD4 T-cell results. This same study also examined Ag-specific T cells stimulated by soluble Ag delivered s.c. in combination with anti-OX40 and/or LPS. Seven days after Ag stimulation anti-OX40 boosted the number of Ag-specific T cells 10-fold compared to controls (Ag + rat Ig) and the anti-OX40/LPS combination increased the numbers 20-fold compared to the control group. Upon inspection of long-term memory (60 days post-immunization) in the soluble Ag model, anti-OX40 increased the number of Ag-specific

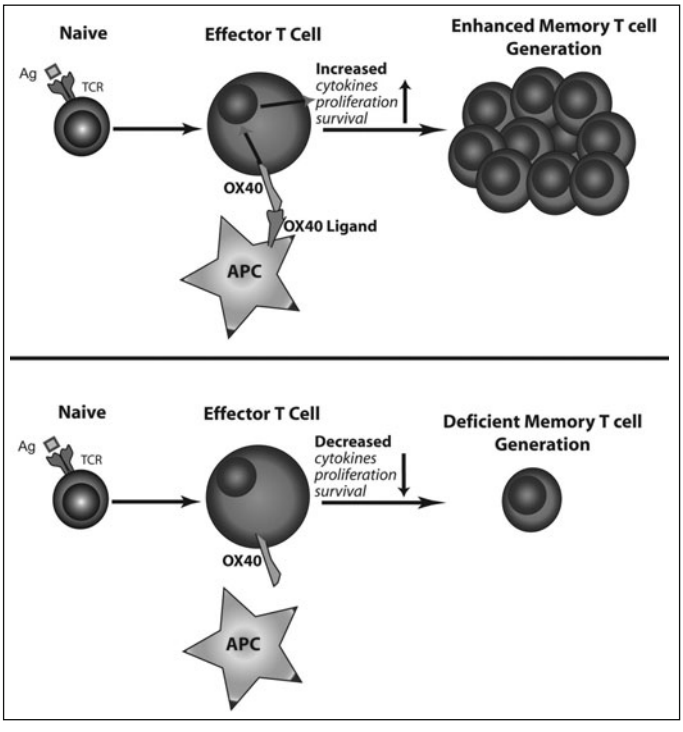

Figure 1. OX40 engagement in vivo enhances effector T-cell function and survival. OX40 ligation through endogenous OX40 ligand expression on antigent presenting cells (APC) stimulates effector T cells, which increases cytokine production, proliferation, and survival leading to increased memory T-cell generation. These properties are observed for both CD4 and CD8 T cells and is dependent OX40 ligand expression, which is only upregulated on activated APC. These same properties can be elicited by providing exogenous signals through OX40 agonists in hosts harboring chronic infections or cancer with positive therapeutic outcomes. Conversely, blocking endogenous OX40 ligation decreases T-cell specific inflammation and has shown great promise for autoimmune disorders.

memory CD4 T cells 15-fold and the anti-OX40/LPS combination increased CD4 T-cell memory survival 70-fold.[3] From this study it is clear that exogenous OX40 stimulation in vivo has potent adjuvant effects leading to increased generation and survival of memory T cells. Ultimately, taking advantage of this type stimulation to increase T-cell memory in hosts harboring cancer or chronic pathogens will be discussed later.

There are a number of T-cell targeted immune adjuvants in the form of soluble Ig fusion proteins and monoclonal Abs.[52] Some of which have potent immune enhancing properties that lead to the eradication of tumors in cancer-bearing mice. Both anti-OX40 and anti-CTLA-4 have anti-tumor efficacy, but mediate their activity through different mechanisms.[52] A side by side comparison of the CD4 T-cell stimulating properties of these two Abs administered in vivo was tested in a soluble Ag immunization model (see Fig. 2). The study showed that both anti-OX40 and anti-CTLA-4 dramatically increased early proliferation of Ag-stimulated CD4 T cells (4 days post-immunization).[4,53] However, the Ag-specific T cells in the anti-CTLA-4 stimulated mice did not survive long-term and return to control levels 10 days after immunization, while the Ag-specific CD4 T cells in anti-OX40 stimulated mice maintained high numbers throughout the course of the experiment. This study also showed that OX40 agonists accentuated Ag-specific Ab responses

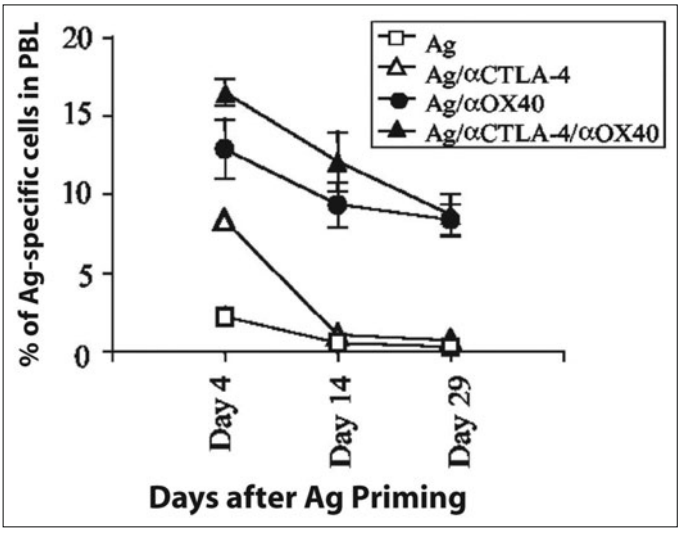

Figure 2. Administration of anti-OX40 after priming with soluble Ag greatly enhances CD4 T-cell survival. Ova-specific naive T cells were transfered into mice and were then immunized with ova (with no adjuvant) with anti-OX40, anti-CTLA-4, or anti-OX40 and anti-CTLA-4. The mice bled on days 4, 14, and 29 and assessed for the percent of ova-specific T cells via the KJ1-26 Ab. the percentage shown in the y-axis is Ag-specific cells of total CD4+ cells and the error bars represents the SE of five mice per group.

in vivo, which was not observed in anti-CTLA-4 treated mice. Subsequent studies showed that anti-OX40 downregulated CTLA-4 expression within Ag-stimulated CD4 T cells during the early priming phase.[53] Anti-OX40-induced CTLA-4 downregulation was shown to be important for OX40 enhanced T-cell proliferation but played no part to enhance memory T-cell survival. A subsequent report identified that a major difference between anti-OX40 and anti-CTLA-4 stimulated T cells was the upregulation of the IL-12 receptor β2 protein (signaling subunit).[54] This report went on to show that anti-OX40-mediated survival of Ag stimulated CD4 T cells was dependent on IL-12 signaling. The data also showed that there was a critical window of IL-12R upregulation, 4-7 days after antigen priming/OX40 stimulation and if the cells did not encounter IL-12 during that time frame they would undergo activation-induced cell death.[54]

Exogenous OX40 agonist administration delivered in vivo also affects CD8 T-cell survival and memory development. The investigators of this study used the OT1 TCR transgenic model (specific for ova) and immunized these mice with soluble ova in combination with anti-OX40 or rat Ig.[5] The anti-OX40 Ab increased the initial expansion phase of Ag stimulated CD8 T cells two-fold compared to the controls. OX40 agonist administration also increased the long-term survival of the CD8 T cells, 5-10-fold. The surviving CD8 T cells were mostly of central memory phenotype and were functional upon re-encountering Ag. The OX40-stimulated CD8 T cells showed a large increase in IL-2 receptor expression early during the response, which might have conferred the increase in their survival. Anti-OX40 administration also greatly enhanced CD8 T-cell recall responses. Both proliferation and survival of the recall specific T cells was increased and was in part CD4-dependent.[5] Although, it is clear that direct expression of OX40 on CD8 T cells also plays a key role for the OX40 agonist effect observed in both primary and secondary responses.[5] OX40 agonists were also shown to increase the effector/cytotoxicity function of antigen stimulated CD8 T cells by upregulating granzyme B levels, through an IL-2-dependent mechanism.[55]

Altering Memory T-Cell Generation/Function through the OX40 Axis for Therapeutic Benefit in Autoimmunity, Cancer and Hosts Harboring Chronic Pathogens

It is clear from the studies summarized above that signaling through OX40 on either CD4 or CD8 T cells has a dramatic effect on their effector function and survival. Hence, research groups have attempted to alter the course of diseases known to have T-cell involvement through either blocking or enhancing OX40 signaling in vivo. What appears to make OX40 such a good target to alter T-cell function in vivo is its unique expression pattern, which is only upregulated after T-cell receptor engagement in vivo and quickly downregulated 24-48 hr after induction. Hence, in vivo expression at any time is extremely low, although OX40 is constitutively expressed on mouse T regulatory cells. The highest expression of OX40 is found at sites of inflammation, such as the colon in inflammatory bowel disease, the CNS in mice with EAE and in tumors/tumor-draining LNs in mice and humans with cancer. $OX40^+$ T cells sorted from these sites of inflammation are enriched for either autoimmune-specific or cancer-specific T cells.[16] Hence, targeting OX40 is a convenient way to home in on the relevant Ag-specific T cells without significantly affecting the peripheral T-cell repertoire.

Two approaches have been used for OX40-specific therapy in autoimmune disease. One involves direct deletion of OX40 positive cells through a cytotoxic Ab, while the other targets the OX40 ligand in attempt to decrease OX40-specific signaling.[15,37] Antibody directed deletion of $OX40^+$ T cells showed therapeutic promise in EAE, as it was able to ameliorate ongoing signs of disease.[15] This was accomplished using a ricin conjugated OX40-specific Ab that was shown to directly target myelin-specific T cells within the CNS of mice with EAE. This therapy led to a 2-log reduction in the myelin-specific T cells isolated from the CNS, which correlated well with a reduction in disease score. While this therapy worked well there has been some concern regarding this approach, because other cell types have more recently been identified to express OX40 including T regulatory cells and PMNs.[10,56] The second approach involves agents that target/bind to the OX40 ligand, thus limiting OX40-specific signals in activated T cells. The OX40 ligand is upregulated at the site of inflammation in several autoimmune models[2] and hence OX40 ligand blockade was a logical extension for treatment of inflammatory disorders. Initial reports showed that injection of an OX40:Ig fusion protein was effective at inhibiting clinical signs of disease in EAE when administered after disease onset.[37] It was also shown that OX40 ligand blockade administered during a relapse episode was effective at tempering disease; however, as soon as treatment was stopped the mice relapsed.[37] Therefore, it appeared that blocking OX40 signaling was able to reduce T-cell effector function, but not eliminate the cells responsible for causing the disease. OX40 ligand blockade has been used to temper a variety of autoimmune/inflammatory models including asthma, inflammatory bowel disease, viral-induced lung inflammation, graft-vs-host disease, diabetes and rheumatoid arthritis.[2,57] Genentech is now developing a humanized OX40 ligand Ab, which is currently being tested in a phase I clinical trial for asthma. This Ab may have far reaching potential as a potent anti-inflammatory for several human diseases in future clinical trials.

Enhancing immune responses through in vivo administration of OX40 agonists has shown therapeutic promise in mouse models for cancer and chronic pathogen infections.[57] Primarily, two agents have been used to achieve successful agonist stimulation: (1) an OX40 ligand:Ig fusion protein and (2) an OX40 agonist Ab. The initial report showed that both OX40L:Ig and anti-OX40 had similar activity to regress tumors in cancer-bearing mice.[14] Although, there has been a more recent report that suggests the OX40L:Ig fusion protein has better anti-tumor efficacy than the anti-mouse OX40 agonist Ab (termed OX86).[58] The OX40-specific anti-tumor efficacy has been observed in several tumor models, including sarcoma, melanoma, colon cancer, breast carcinoma, lung cancer, glioma, prostate cancer.[2] The anti-tumor efficacy generated by OX40 agonists is dependent on both CD4 and CD8 T cells and it has been shown that OX40 agonists do enhance tumor-Ag specific memory T-cell development.[14,59] Subsequently, another report has shown that anti-OX40 administration greatly augments the adoptive transfer of tumor-reactive

Table 1. Potential use for OX40-specific treatment in disease

	Treatment Schemes	
	OX40 Agonists	**OX40L Blockade**
Agents used	Anti-OX40 mAb OX40L-lg fusion protein OX40L-transduced/transfected APCs Recombinant OX40L-expressing virus/tumor/bacteria OX40-specific DNA aptamers	Anti-OX40 ligand mAb OX40-lg fusion protein
Potential diseases for therapeutic use	Cancer (all types) Persistent bacterial infections Chronic viral infections (HIV, hepatits C)	Multiple sclerosis Rheumatoid arthritis Allergic asthma Inflammatory bowel disease Lupus Type 1 diabetes Psoriasis Atherosclerosis GVHD Pathogen-induced inflammation (influenza, SARS, West Nile virus)

T cells.[60] Anti-OX40 showed similar therapeutic efficacy to IL-2 in supporting tumor-reactive T-cell mediated destruction of lung-metastases. However, IL-2 in combination with adoptive immunotherapy did not support the eradication of brain metastases, while anti-OX40 showed powerful synergy to eradicate brain metastases.[60] It was not clear why anti-OX40 was able to augment the efficacy of T cells while IL-2 was not, but it may be linked to differential expression of T-cell surface proteins and their ability to help break the blood brain barrier.

While it is clear that OX40 agonists given as a single agent can enhance anti-tumor immunity in cancer-bearing hosts, there are models where its activity alone is not enough to cure mice of disease. Hence, there have been a number of studies that have attempted combination therapies with vaccines/cytokines and anti-OX40.[54,61,62] These combination therapies have included GM-CSF secreting whole tumor vaccines as well as the addition of innate cytokines, both of which showed promising synergy.[54,61,62] It was shown that anti-OX40 in combination with a GM-CSF secreting whole cell vaccine expressing the Her-2/neu tumor Ag was able to enhance CD8 T-cell responses and regress tumors (breast cancer model).[62] However, the vaccine alone showed very little anti-tumor efficacy, which correlated with a weak Her-2-specific CD8 T-cell response. It was subsequently shown that the increase in Her-2-specific CD8 T cells elicited via the combination treatment was dependent on anti-OX40 accentuating CD4 T-cell help. Another combinatorial approach that has shown great promise for tumor immunotherapy is combining anti-OX40 with innate cytokine(s), especially IL-12. One of the theoretical limitations of priming the immune system to tumor-specific Ags is the lack of "danger" signals (e.g., CpG, dsRNA, LPS, etc...) known to elicit innate cytokines when tumor Ags are presented to the immune system in vivo. As previously mentioned, IL-12 is necessary to mediate the survival of anti-OX40-stimulated CD4 T cells and the combination of anti-OX40 and IL-12 in tumor-bearing mice showed synergistic therapy.[54] This combination was therapeutically effective in the poorly immunogenic prostate cancer model, TRAMP-C1, where neither IL-12 nor anti-OX40 alone showed any therapeutic efficacy.[54] IL-12 and anti-OX40 also showed dramatic therapeutic synergy in an active immunization model using a tumor-dendritic fusion

vaccine injected directly into the spleen.[54] Whether IL-12 actually enhances survival of OX40 stimulated tumor-reactive T cells or increases Th1/Tc1 immunity was not directly ascertained in these models, but most likely both mechanisms are involved to enhance OX40-mediated tumor destruction.

OX40 agonists have also been shown to enhance T-cell responses to chronic pathogens (e.g., viruses and bacteria). In particular, anti-OX40 was administered into mice harboring a chronic cytomegalovirus known to replicate in visceral organs (i.e., salivary gland).[63] The initial study showed that anti-OX40 enhanced viral specific effector T-cell differentiation leading decreased viral replication in the salivary gland. Subsequently this group treated mice during the initial stage of infection and found that anti-OX40 greatly enhanced CD8 T-cell responses during the early stages of infection, which led to protective immunity.[64] The OX40 agonist strategy was also beneficial against hosts infected with Cryptococcus neoformans, where the infection resides in the lungs and becomes persistent.[65] The persistence of this pathogen is characterized by immune deviation to a nonclearing Th2 response, leading to chronic eosinphilia in the lungs.[65] Administration of an OX40L:Ig fusion protein drove a cytokine switch from Th2 to Th1 and reduced the pathogen burden and reduced the eosinphilia. OX40-specific elimination of C. neoformans was dependent on IFN-γ/IL-12, as injection of an OX40 agonist to IFN-γ or IL-12 ko mice harboring the pathogen was not able to resolve the infection.

It is clear that OX40 agonists have potent immune stimulating properties in several disease models, which ultimately helps the host eradicate harmful/potentially lethal entities within the body. Recently, our group has translated these findings into a cancer patient-specific clinical trial. Initially we tested the safety/dosing of a mouse anti-human OX40-specific monoclonal Ab in nonhuman primates.[66] We found that OX40 agonist administration to nonhuman primates potentiated memory T-cell generation and increased Ag-specific Ab responses similar to what was observed in mice. However, in contrast to the mouse studies we found that the adjuvant affect lasted longer, up to a month after the injection. We also observed a transient in drop in lymphocyte counts in the peripheral blood seven days after the initial Ab infusion and this was followed by a rebound where the lymphocyte numbers increased over base-line values. Upon completion of the monkey studies, the FDA approved a Phase I clinical trial in patients with Stage IV cancer. So far we have treated 17 patients with relatively low toxicity and we have observed immune stimulatory effects in most of the patients post-anti-OX40 treatment. In particular we have observed an increase in cycling memory T cells starting with the CD4 T-cell population one week after Ab infusion, followed by CD8 T cells usually 2-4 weeks post-OX40 treatment. We have observed cycling T cells in both the central memory compartment ($CD28^+$) and effector memory ($CD28^-$) populations. The increase in cycling memory T cells usually lasts 28-days following Ab infusion, however in some patients this effect lasted during the entire two-month evaluation period. There has been some hint of anti-tumor activity with four patients showing regression of some metastatic disease; however there have been no complete responders on this trial to date.

Conclusion

In summary, OX40-specific signaling within T cells plays a key role in the generation of memory T cells as well as T-cell effector function. Its biologic function in vivo appears to be limited by expression of the OX40 ligand, which is expressed mainly on activated antigen presenting cells. OX40 ligand expressing cells are found within sites of inflammation in autoimmune disease and hence strategies have evolved to temper inflammation via blockade of OX40/OX40 ligand interaction. In contrast, little to no OX40 ligand expression is observed in hosts harboring tumors and some chronic infections and therefore accentuating OX40 signaling can enhance immunity leading to the destruction of these harmful entities. It is clear that tipping the balance of T-cell immunity through the OX40 axis could have important ramifications for several human diseases. The first stages of OX40-specific clinical trials are now being performed and efficacy of these trials will be determined in the future.

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Chapter 6

The Role of Precursor Frequency in the Differentiation of Memory T Cells:
Memory by Numbers

Amanda L. Marzo,* Ryan T. Sowell and Bernadette Scott

Abstract

Immunological memory is considered the hallmark of adaptive, or acquired, immunity. That ability of our immune system to recognize and respond to those pathogens we have encountered before not only typifies acquired immunity but has provided the basis for the most notable of medical interventions: vaccination. Yet, as much as we now know about this process, we are still on the cusp of fully understanding how memory T cells develop, how they are maintained and the importance of memory T-cell heterogeneity. In this review we will primarily focus on our understanding of CD8 T-cell memory generated during acute infections and how precursor frequency influences their development and functional attributes.

History of Immunological Memory

The nascence of our Western understanding of immunological memory originates from the inoculations undertaken by Jenner in the 18th century to prevent smallpox[1] and the subsequent broadening of the concept by Pasteur in his germ theory of disease in the 19th century.[2] However these great experimentalists were the heirs of knowledge belonging to Indian and Chinese physicians of the early 11th century. These practitioners used material from infected patients to inoculate healthy individuals against smallpox thereby demonstrating their knowledge that previous exposure to a disease was protective. Even more remarkable, was the realization that the survivors of a disease were unlikely to succumb to reinfection had been made as early as the 5th century B.C. Thucydides, the chronicler of the great plague of Athens during the Peloponnesian War, wrote that those that survived the "plague" tended the sick because "no one was ever attacked a second time, or not with a fatal result".[3]

Elucidating our modern concepts of immunological memory first required an understanding of the cellular nature of adaptive immunity. The development of theories concerning the clonal nature of the immune system, such as those described by Niels Jerne[4] and MacFarlane Burnett[5] amongst others, as well as Jacque Miller's experiments and insights into the role of the thymus[6,7] in the mid 20th century, paved the way for experimentation into the mechanisms of underlying immunity, response maturation and memory development. Today we know that immunological memory is a characteristic of both B- and T cells and is, not unexpectedly, complex.

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Inroads into Understanding T Memory Development

T-cell memory is functionally defined as long–lived recall responses and is a well-established concept of modern immunology. At a cellular level, memory is established in several phases. T cells become activated after encounter with antigen and then undergo a massive clonal expansion. In this period they acquire the ability to kill and produce cytokines, resulting in a population of effector cytotoxic T-lymphocytes (CTLs) that disseminate throughout the body to eradicate the pathogen. Subsequently, a dramatic contraction phase occurs, where most of the effector CTLs die. However, a numerically small but stable population of T cells survive the contraction leading to a distinct population of apparently long-lived T cells (Fig. 1).[8-12] These long-lived cells respond to rechallenge more quickly, more efficiently than observed as a result of the initial antigen encounter.

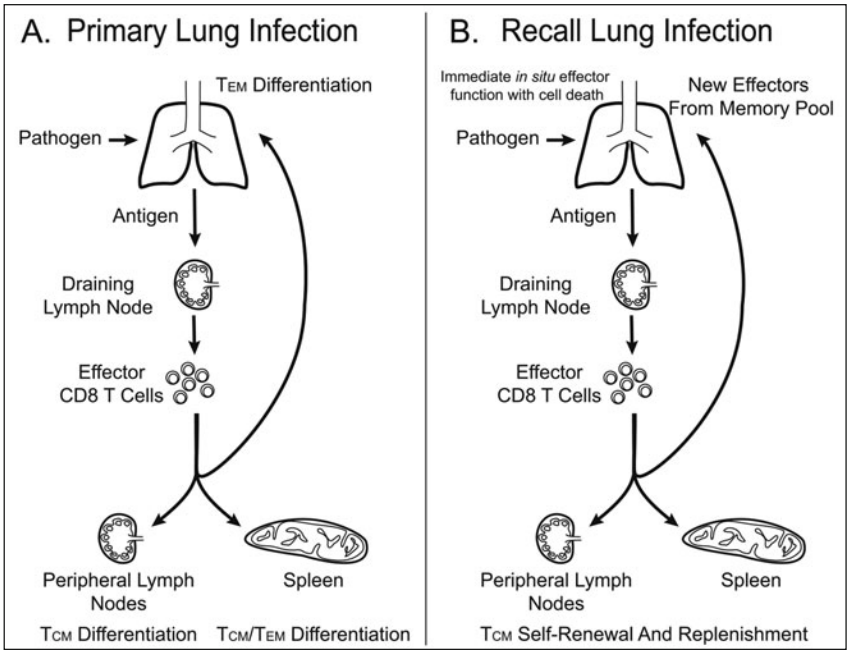

Figure 1. Differentiation of primary and secondary memory CD8 T cells. A) In a primary response such as an insult with a pathogen that infects the lung, antigen is taken to the draining lymph node where naive antigen specific T cells become activated and expand to competent effectors. These cells down-regulate lymph node homing receptors (CCR7 and CD62L) and then migrate throughout the body populating both secondary lymphoid tissues such as the nondraining lymph nodes, spleen and various nonlymphoid tissue including the lung where the infection originated. In acute infections these cells succeed in clearing the virus. Subsequently, a contraction phase occurs and T cells that survive this phase are termed memory T cells. Memory T cells that reside in the lung predominately posses the T_{EM} phenotype (CCR7-/CD62L-) in contrast, T_{CM} predominate in the lymph nodes and are characterized by up-regulation of the lymph node homing receptors CCR7 and CD62L. The spleen 30 days after infection is dominated by T_{EM} however, T_{CM} slowly accumulate in number with time. This is because T_{CM} have a greater capacity to self renew compared to T_{EM}, via a process of homeostatic proliferation.[37,36] B) In a recall response T_{EM} already residing in the lung are poised for controlling a further insult. Their capacity for immediate effector function facilitates the elimination of the pathogen. In addition, T_{CM} and T_{EM} also residing in the spleen contribute to a higher precursor of antigen specific cells that can be activated upon rechallenge and subsequently disseminate throughout the body, including the lung and aid in pathogen eradication. Thus this process results in new effectors being generated from both the memory T-cell pool and naive T cells specific for the pathogen.

Our ability to specifically identify memory T cells began with studies of Peter Beverley and colleagues who recognized that two monoclonal antibodies, CD45RO and CD45RA, were expressed on reciprocal populations of human T cells with differing functional capacities; memory and naive T cells respectively.[6,13,14] Later, Sallusto and colleagues showed that memory T cells could be further divided into at least 2 functional subpopulations that differentially expressed the chemokine receptor 7 (CCR7). Specifically they demonstrated that one of these T-cell populations that survived the initial contraction phase did not, for the most part, express CCR7. These $CCR7^-$ cells were generally excluded from lymphoid tissues, preferring to migrate into peripheral organs[15-17] and thus coined effector memory T cells (T_{EM}). T_{EM} express both perforin and granzyme B[16] and are capable of direct ex vivo lytic activity.[18] The second population of memory T cells that survived re-expressed the lymph node homing receptors CCR7 and CD62L, thereby facilitating their recirculation and re-entry into lymphoid tissues. In contrast to T_{EM}, this second memory T-cell population lacked immediate cytolytic function. These recirculating memory T cells were termed central memory T cells (T_{CM}). In addition to the expression of CD62L and CCR7, T_{CM} were later found to express CD27, a member of the tumor necrosis factor (TNF) receptor superfamily[19,20] and CD127, the α chain of the IL-7 receptor.[21] T_{EM} on the other hand lacked expression of CCR7, CD62L and CD27 while they were found to retain expression of CD127.

The studies above have provided a means to define different memory populations but the question of what shapes this process and how memory is maintained continues to be the subject of intense research. One of the parameters considered to be a critical factor in shaping the memory T-cell response was the persistence of antigen though this was a matter of intense debate. Evidence cited to support the requirement for antigen persistence included human vaccinations which required booster immunizations to reinvigorate flagging immunity. In mouse models, early viral studies showed that protection to virus declined with time[22] and studies measuring the response to the male antigen H-Y[23] also supported this premise. However subsequent experiments, utilizing the capacity to better phenotype memory T cells, showed that the maintenance of T-cell memory was not dependent on the persistence of antigen. Studies using purified memory CD8 T cells isolated from Lymphocytic Choriomeningitis Virus (LCMV) immune mice and adoptively transferred into naïve recipients found that these memory CD8 T cells to survive long-term. In addition, these long-lived memory CD8 T cells were also able to provide protection against virus challenge.[24] Similar experiments using Sendai Virus demonstrated that Sendai specific memory CD8 T cells were also maintained in the absence of antigen.[25] In order to try and understand the discrepancies observed between experimental systems, Wherry and colleagues investigated the effect of antigen persistence in acute compared with chronic LCMV infections.[26] They showed that antigen clearance, as seen in acute viral infections, leads to "typical" memory T cells that survive in the absence of antigen. They were also able to isolate memory T cells from hosts with chronic LCMV infections, (i.e., antigen persistence). However, in contrast to memory T cells isolated from acute infections, the memory T cells induced during chronic infections were unable to survive if transferred into uninfected, secondary recipients i.e., these cells did not survive in the absence of antigen.[26] These data were interpreted to mean that the duration of antigen encounter, brief in the case of an acute infection or persistent for chronic infections, influenced the type of memory T cell that developed. More recent data suggest this may not be a universal rule. Mice infected with *Trypanosoma cruzi* (*T. cruzi*) cannot clear the parasite, resulting in chronicity. However, unlike LMCV, there is a population of T_{CM} that survive without encountering persistent antigen. In this case there is an anatomical division as to where antigen persists and where the T_{CM} reside. In *T. cruzi* infection, low levels of antigen persist in muscle, adipose and neural tissues.[27] Given that *T. cruzi* specific T_{CM} are largely excluded from peripheral tissues encounter with antigen is unlikely, although one cannot rule out the possibility that these T_{CM} are not replenished by newly recruited cells from the naïve population.[28] However, upon transfer into naïve recipients, these *T. cruzi* specific T_{CM} were capable of antigen independent survival for at least 20 days.[27] What these data emphasize is that context is an important defining parameter in memory T-cell differentiation.

Use of TCR Transgenics to Elucidate T-Cell Biology

The use of T-cell receptor (TCR) transgenic mice revolutionized our capacity to study T-cell function. One of the reasons for the use of TCR transgenic T cells to study T-cell responses is that they enable us to study antigen specific T cells at a frequency that facilitates experimentation. Using this technology important progress was made into our understanding of T-cell development,[29,30] central[31] and peripheral tolerance induction[32,33] and how T cells can interact with specific antigen in vivo.[34,35] Indeed, such technology has been used in almost every type of immunological system, including studies into memory T-cell differentiation.

Despite the increased ability to phenotype memory T cells, the pathways and relationship between the various stages of T-cell differentiation, as well as the signals that drive the process, have not been well understood. In terms of differentiation pathways, the original experiments of Sallusto et al were consistent with a linear model of memory T-cell differentiation in which T_{CM} function as precursors of T_{EM} thus providing a continual source of T_{EM} (Fig. 2A). However, this in vitro model has not been supported by in vivo data.[36,37] In order to investigate the relationship between T_{CM} and T_{EM}, Wherry et al[36] made use of TCR transgenic technology to facilitate analysis of memory T-cell differentiation. They used the well characterized P14 TCR transgenic mice, which recognize the H-2^b restricted epitope of LCMV gp33, in their experiments in order to facilitate identification of antigen specific cells in vivo. In these elegant experiments, as with others before and since, high numbers of naive P14 TCR transgenic T cells, typically ~7×10^4, were transferred into naïve, secondary, recipients to produce "chimeras". These chimeras were subsequently infected with LCMV and the phenotype and function of resultant T_{EM} and T_{CM} compared and contrasted. As previously described for endogenous T memory responses, they showed that the transferred TCR transgenic cells became long-lived memory CD8 T-cell populations i.e., the T cells could be identified at least 30 days post infection and be divided into those that expressed high levels of CD62L and CCR7 and those that did not. Importantly this dichotomy also segregated with other previously defined markers of T_{CM} and T_{EM} subpopulations. In the studies described by Wherry et al, the CD62L expressing cells also expressed CCR7 and high levels of CD27. Conversely, $CD62L^-$ cells did not express CCR7 and little if any CD27. They also showed that both memory T-cell populations were functionally different. Though both T_{EM} and T_{CM} were able to produce effector molecules (IFN-γ and TNF-α), the T_{EM} population was deficient in IL-2 production upon restimulation in vitro. In vivo, this difference in functional capacity was also seen in the relative ability of the two subpopulations to control viremia on a cell for cell basis. Again, using adoptive transfer techniques, when T_{EM} or T_{CM} were adoptively transferred into naive recipients and then rechallenged, T_{CM} mediated a more effective reduction of virus than transferred T_{EM}, irrespective of route of administration for the infectious agent. This enhanced anti-viral function was associated with a greater proliferative capacity of T_{CM} in vitro or in vivo. The authors also showed that the majority of long-lived memory T cells were predominantly CD62L high and not only could their persistence be explained by an enhanced proliferative potential, but from conversion of $CD62L^-$ cells to CD62L expressing cells. In fact, when the two populations were monitored over long periods of time, there was a decline in the T_{EM} population with a concurrent rise in the T_{CM} cells, implying a linear differentiation relationship between these two memory T-cell types (Fig. 2B).

Precursor Frequency Affects Memory T-Cell Development

Despite what appeared to be strong evidence for this linear relationship, older studies that were consistent with other models still needed to be reconciled. The early work of Sallusto and Lanzavecchia suggested,[16] in direct contrast to the transgenic studies of Wherry et al[36] described above, that T_{CM} converted into T_{EM} as the T_{CM} cells lost CCR7 expression and gained functional capacity in vitro (Fig. 2A). On the other hand, a third model proposed by Baron et al, analyzing TCR usage in human memory T cells, found that the TCR usage was unique and implied that these populations were distinct lineages (Fig. 2C).[38] What is the source of these discrepancies? Is it a species-specific problem? Or is it the result of the systems used?

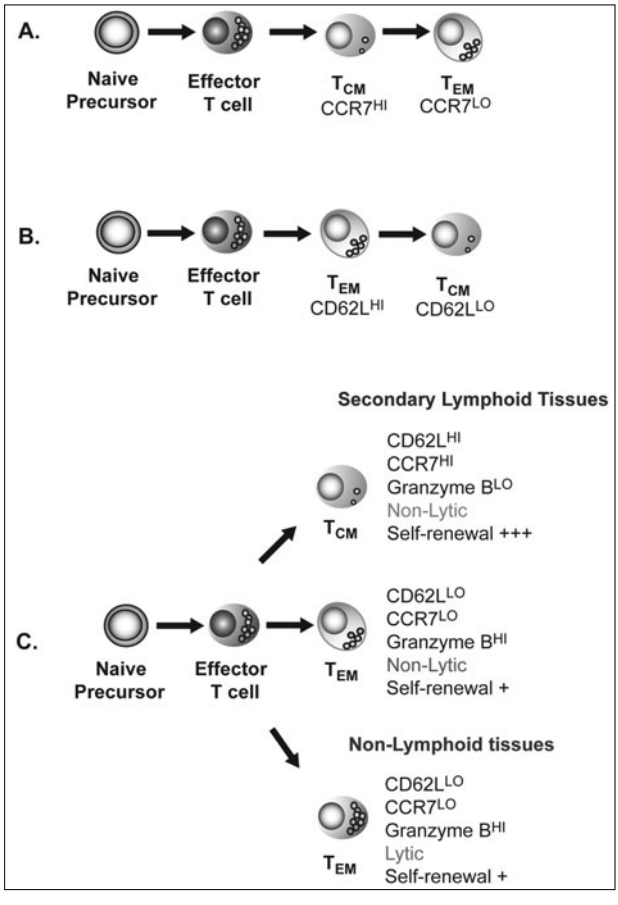

Figure 2. Models of memory T-cell development. A) T_+ produce T_{EM}. This model demonstrates a linear pathway of memory T-cell development. According to this model T_{CM} are generated from effector T cells and are capable of self renewal. T_{CM} also give rise to more terminally differentiated T_{EM}. The model was based on in vitro data where they culture T_{CM} and found that after time they had both T_{CM} that were CCR7+ and CD62L+ and T_{EM} that lacked both CCR7 and CD62L. B) T_{EM} convert to T_{CM}. This model was generated using high number of transgenic T cells and demonstrates that T_{EM} are lost over time while T_{CM} accumulate in number. The model predicts that T_{EM} actually convert to T_{CM} and then increase over time, although whether these T_{EM} actually convert or are lost is still debated. C) T_{CM} and T_{EM} develop from separate lineages. In this scenario both T_{CM} and T_{EM} are derived from effectors as separate but stable lineages and are maintained by differential self-renewal capacities. With T_{CM} increasing in number compared to T_{EM} over time. It has also become evident that T_{EM} that reside in nonlymphoid tissues such as the lung are functionally more superior to those that reside in secondary lymphoid tissues such as the spleen.[18]

Later studies pointed towards the conclusion that the system utilized influenced the experimental outcome, more specifically that the use of TCR transgenic cells skewed the biological outcome.[37] It was found that memory T-cell development in adoptive transfer models did not mirror that of the endogenous response. In these experiments, like those of Wherry et al, adoptive transfer of large numbers of naive TCR transgenic T cells (10^4-10^7 cells) into naive recipients, subsequently infected them with virus, lead to a T_{EM} to T_{CM} conversion. However, concomitant analysis of the

endogenous T-cell response showed a different profile of memory T development. In the endogenous response, though both T_{EM} and T_{CM} were generated there was not a linear relationship between the two populations. T_{EM} were a stable population that did not differentiate into T_{CM}. These data imply that the precursor frequency of the initial responder cells shapes the composition of the memory response that develops. In support of this conclusion, when small numbers (5×10^2) of transgenic CD8 T cells were transferred, the response was similar to that observed for the endogenous response. Furthermore, this stability of memory T-cell phenotype was observed irrespective of the infectious agent (LCMV or Vesicular Stomatitis Virus, VSV) or of the antigenic epitope monitored (gp33 or OVA) when initial responder numbers approximated physiological frequencies. Moreover, these separate lineages were "imprinted" during the primary infections because a secondary challenge did not alter their phenotype. These experiments were supported by those of Badovinac et al, who, undertook similar titration experiments of TCR transgenic T cells, using recombinant Listeria monocytogenes (LM), expressing ovalbumin (OVA), as the infectious agent and OT-1 T cells as responders.[39] They also demonstrated that transfer of large numbers of TCR transgenic T cells skewed the distribution of the memory T-cell populations.

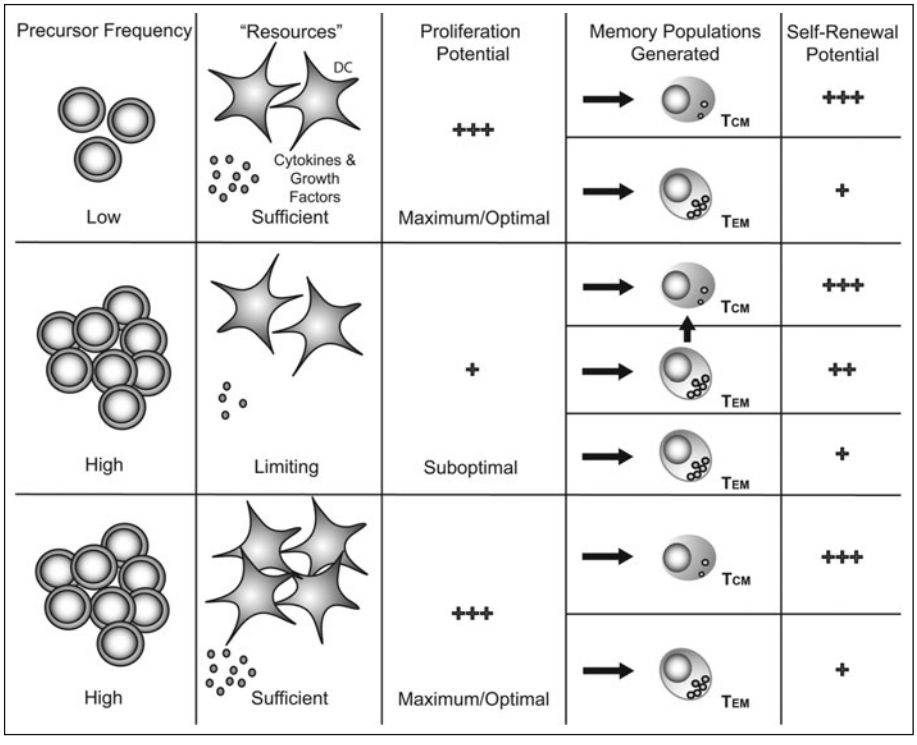

Figure 3. Model of T_{CM} and T_{EM} differentiation. The model depicts three scenarios where T_{CM} and T_{EM} are generated where (A) low precursor frequency of naïve T cells get activated in the presence of sufficient resources resulting in the generation of both T_{CM} and T_{EM}, (B) high precursor frequency of naïve T cells get activated in the presence of limiting resources resulting in sub-optimal priming and the formation of both T_{EM} and T_{CM}. In addition a third population of transitional T_{EM} develop that convert to T_{CM} and (C) high precursor frequency of naïve T cells activated in the presence of sufficient resources results in an optimal response and the generation of both T_{CM} and T_{EM} memory subsets. We hypothesize another consequence of high precursor frequency and limiting resources is that the effector T cells will undergo sub-optimal proliferation contributing to the altered T_{EM} differentiation and the generation of transitional T_{EM}.

Another consequence of supraphysiological numbers of primary responders was that the peak of the response shifted forward in time. The enhanced production of T_{EM} over that of T_{CM} cells was also observed when the potential for antigen presentation was increased by treating mice with FLT3L, thereby increasing dendritic cell (DC) numbers.[37] These data are most consistent with that of the Baron model of distinct memory T-cell lineages and non conversion and clearly demonstrate that the initial precursor frequency impacts on the type of memory T-cell response evoked.[37,39] The question is how does the initial frequency of T cells alter the long-term functional makeup of the T-cell memory compartment? Together, these data imply that at least one of the parameters that drives T_{CM} or T_{EM} differentiation is the availability of resources (Fig. 3). When there are too many responders to be efficiently processed through the system, as is the case when large numbers of TCR transgenic T cells are transferred, T_{CM} production is favored. In contrast, when resources are not limiting i.e., at low precursor frequencies or when antigen presentation is not limiting, T_{EM} production is favored. However, other factors could impact the outcome of T memory differentiation. When the precursor frequency is high there may be a reduction in the number of cell divisions. With a reduction in cell division one could hypothesize that the resultant memory T cells would be of a less differentiated phenotype. This could potentially result in a "transitional" memory T cell that initially has a T_{EM} phenotype but, over time, converts to a T_{CM} phenotype (Fig. 3).

For decades now a question that has remained largely theoretical has been what is the precursor frequency of T cells for their cognate epitope? Tetramer technology has provided the answer for a number of epitopes for both CD4 and CD8 T cells. Moon et al studied three CD4 epitopes and determined the naïve precursor frequency for W1S:I-A^b epitope, FliC:I-A^b and Ova:I-A^b to be 200, 20 and 20 cells, respectively.[40] Another group later examined six CD8 specificities and showed a range of 80-1200 cells/per mouse (with an average of 120- 600).[41] These data confirm that the traditional methods of adoptively transferring large number of TCR transgenic cells are orders of magnitude above physiological frequencies.

One Cell, Many Fates

As there are still competing models of memory T-cell differentiation, further studies looking at the underlying mechanisms that control the cellular transitions are needed. Recent experiments by Stemberger et al, demonstrated that the adoptive transfer of a single CD8 TCR transgenic T cell, followed by infection, gave rise to all the phenotypic and functional CD8 T-cell subsets observed in the endogenous repertoire from effector to memory T cells.[42] It is remarkable that a single cell has the potential to become both subsets of memory T cells (T_{CM} and T_{EM}). Furthermore, they showed that the relative composition of the T-cell memory response i.e., the proportion of T_{CM} and T_{EM}, differed with different modes of insult. Given the reproducibility of the differentiation process in these experiments, the model of differentiation that could be applied would be one that was instructional. That is, in these single cell experiments, T-cell memory differentiation is not hard wired but responds according to how the pathogen is encountered and the coordinated response that ensues. These results are consistent with the idea that memory T-cell development is driven by encounter with antigen and the resultant contribution of T_{EM} and T_{CM} depends on the type of pathogen and the tissue in which the response is primed. Such a mechanism may facilitate the response upon reencounter with the pathogen and ensure that the most suitable response is engaged. Whilst these experiments show that T-cell memory differentiation is not predetermined, the difficult question is what cellular mechanisms could account for this capability? The answer may lie, in part, in the use of an evolutionarily conserved process, asymmetric cell division.[43] This cell-intrinsic process is used to create two daughter cells of differential potential from a single precursor. Recent data has demonstrated this phenomenon in T cells.[44] Using confocal microscopy, Chang and colleagues demonstrated that in activated T cells that had not yet divided, signaling components, such as CD3, CD4 or CD8, were polarized perpendicular to the mitotic spindle, a morphological characteristic of asymmetrical cell division. It was shown that an antigen-APC interaction was required for this phenomenon as it did not occur in T cells

that had initiated homeostatic proliferation. Importantly, proteins that have been established as components of asymmetrical cell division in other systems were also segregated appropriately in these T cells. Asymmetric cell division provides an attractive mechanism whereby a single T cell could give rise to progeny with different functional capacities. Importantly Chang et al went one step further and showed that the proximal daughter cells of the activated T cells expressed features and markers consistent with effector T cells. On the other hand, the distal daughter cells, defined as those that were further from the immunological synapse, expressed markers consistent with memory T cells. Even more remarkably, when sorted and transferred into naive recipients, upon rechallenge with the bacterial pathogen LM at 30 days post transfer, the putative memory cells (i.e., the distal daughters) had a greater protective function than the proximal daughter cells, though both were equally effective at reducing the bacteria burden in an acute assay. These results are consistent with the known properties of memory (renewal, survival and long-lived protective capacity) and effector (limited renewal, short-lived protective capacity) T cells. What is important to note in these experiments, is that the data is derived from an initial transfer of high numbers (10^7) of transgenic T cells. Indeed, the phenotype of the memory T cells derived in these transfer experiments was consistent with a T_{CM} phenotype (i.e., the predominant phenotype observed in the earlier memory studies using adoptive transfer of high numbers of TCR transgenic). Further studies are required to unravel this problem. Given that processes like asymmetric cell division and epigenetic modifications are well-described mechanisms for shaping the fate or function of cells in other systems, it is not unlikely that they could be utilized by the adaptive immune system to direct memory development.

Which Are the Better Protectors?

Why do we worry about understanding the differentiation of T cells into various memory populations? It is because this heterogeneity implies distinct functional capacities and therefore, potentially distinct protective capacities. Unfortunately there is no consensus as to which of the broad T memory populations provide superior protection when re-encountering pathogen. In the acute studies with LMCV, T_{CM} were shown to be more efficient than T_{EM} at clearing secondary viral infections.[36] Studies using Sendai virus demonstrate that early after infection the recall response relies predominately on T_{EM}, however at later time points T_{CM} dominate.[45,46] This change in relative contributions of the T_{EM} and T_{CM} is probably a consequence of the increased proliferative capacity of the T_{CM} compare to T_{EM}.[36,37] It is probable that the most appropriate memory T-cell response will depend on the pathogen itself and the site of priming and the tissue in which they reside. To begin to address this issue Klonowski et al used lymphotoxin α (LTα) null mice (which lack lymph nodes) as well as LTαsplenectomized mice to evaluate how priming in the recall response affects existing T-cell memory. Perhaps not altogether surprisingly, they demonstrated that the memory T-cell response to infectious agents, either recombinant LM-OVA or VSV, required secondary lymphoid tissue. Antigen independent proliferation, as occurs with homeostatic proliferation, was not dependent on lymph nodes or spleen. More specifically, LM-OVA memory cells required the presence of the spleen and T_{EM} and T_{CM} populations responded with nearly identical efficacy to the pathogen. VSV infection differed in that it was lymph node dependent and T_{CM} were able to respond more efficiently to the virus than T_{EM}. Overall these data suggest that both the location of priming and the location of T_{CM} and T_{EM} determine which memory T-cell subset participates in the recall response. However, it should be noted that increased numbers of memory cells does not necessarily mean better protection.

Conclusion

The observation that the use of high numbers of transgenic T cells does not necessarily reflect the endogenous response is not the end of their use in studying immunological phenomenon. It is partly a reminder, that not all systems are perfect and require verification by other means. In addition we can take the opportunity to make use of these data to our advantage. For example, adoptive therapy is a strategy that has been trialed in cancer treatments and is exemplified by trials

in the treatment of melanoma. There has been some progress, but clearly not enough for translation to a more widespread clinical application. Perhaps the study of adoptive transfer and memory development is the aspect of treatment that will prove to be pivotal.

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Chapter 7

CD8 T-Cell Memory Differentiation during Acute and Chronic Viral Infections

Vandana Kalia,[‡] Surojit Sarkar[‡] and Rafi Ahmed*

Abstract

CD8 T-cell responses play an important role in protection against intracellular pathogens. Memory CD8 T cells mediate rapid clearance of pathogens upon secondary infection owing to their elevated frequency, ready localization to peripheral sites of infection and their ability to rapidly expand and mount effector functions. Such potent long-lasting protective memory CD8 T cells develop in acute infections where antigen is effectively cleared. In contrast, chronic infections with persistently high viral loads are characterized by CD8 T-cell dysfunction. In this chapter we present our current understanding of signals and mechanisms that regulate the development of functional long-lived memory CD8 T cells during acute infections. This is discussed in the context of proposed models of memory differentiation and compared with CD8 T-cell exhaustion and altered T-cell homeostasis, as occurs during persistent viral infections.

Introduction

Immunological memory is a cardinal feature of adaptive immunity, whereby the first encounter with a pathogen is imprinted indelibly into the immune system. Subsequent exposure to the same pathogen then results in accelerated, more robust immune responses that either prevent reinfection or significantly reduce the severity of clinical disease. Protective immune memory can persist for many years after initial antigenic exposure, even up to the lifetime of an individual. Both humoral and cellular immune responses comprise important arms of immunological memory and have evolved to perform distinct complementary effector functions of tackling free pathogens versus infected cells. Humoral immunity includes preexisting antibody, memory B-cells and long-lived plasma cells. The antibodies provide the first line of defense by neutralizing or opsonizing free extracellular pathogens. CD4 T cells further provide help for antibody production and the generation and maintenance of CD8 T-cell memory. Memory CD8 T cells, unlike antibodies, cannot recognize free pathogens, but instead identify infected cells and exert effector functions including direct cytotoxic effects on target cells and/or release of cytokines to inhibit growth or survival of the pathogen. Thus, the development of CD8 T-cell responses is necessary for the control of a variety of intracellular bacterial and viral infections and tumors.

In this chapter, we will focus on our current understanding of how protective CD8 T-cell responses are generated and maintained following two major types of infection, acute and chronic. Viral infection is largely divided into two types: (i) acute, where virus is eliminated;

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and (ii) chronic, where virus persists. Chronic infections are further classified into two broad categories: (i) latent infections, where virus is usually dormant, but occasional viral replication may occur during periodic episodes of reactivation; and (ii) persistent infections, where viral replication continues to maintain persistent viremia. Acute viral infections usually result in effective antiviral immune responses. In contrast, chronic persistent infections are typically associated with compromised CD8 T-cell function. In this chapter we will first focus on CD8 T-cell immunological memory following acute infections and discuss the underlying mechanisms of memory CD8 T-cell differentiation and maintenance when antigen is effectively cleared from the system. We will then discuss how the CD8 T-cell differentiation program is altered during chronic infections, where viral loads are maintained at persistently high levels. Due to the plethora of information pertinent to this topic, we will primarily describe broad themes of CD8 T-cell differentiation that have emerged from studies in the mouse model system, with appropriate references to other higher order model systems, which are discussed extensively elsewhere.

CD8 T-Cell Responses following Acute Infection

Primary infection results in the activation and proliferation of a subset of naïve CD8 T cells that have the capability of specifically responding to the invading pathogen. By some estimates, a mouse contains 50-200 naïve CD8 T cells specific for any one epitope.[1] Following activation, naïve cells go through as many as 15-20 cell divisions and expand their numbers by up to 50,000 fold.[2-4] At the population level, these expanded CD8 T cells express effector molecules such as perforin, granzymes and antiviral cytokines that aid in the elimination of infected host cells and are typically referred to as cytotoxic T-lymphocytes (CTL). After clearance of the pathogen, most pathogen-specific CD8 T cells die, but a small fraction (5-10%) of the cells survive long-term and form the memory pool of CD8 T cells (Fig. 1), which provides rapid protection to the host in case of reinfection with the pathogen (Table 1).[5,6]

A typical primary CD8 T-cell response to acute infection is classically divided into three phases based on the kinetics of accumulation of antigen-specific T cells, as well as specific functional and phenotypic properties that are associated with them (Fig. 1): (i) the effector phase, when naïve CD8 T cells get primed, undergo dramatic expansion, differentiate into potent killer cells (cytotoxic T-lymphocytes, CTL) by acquiring a host of effector functions (such as: antiviral cytokine production, cytotoxicity, chemokine production and the ability to migrate to peripheral sites of infection to mediate pathogen clearance); (ii) the contraction phase, when majority of the effector CD8 T-cell population dies, with about 5-10% cells of the original burst size surviving to form the long-lived memory pool; (iii) the memory differentiation and maintenance phase, when surviving CD8 T cells progressively acquire hallmark memory characteristics and stable memory CD8 T-cell numbers are maintained via homeostatic proliferation for up to the lifetime of the mouse. Memory T cells differ from both naïve and effector T cells and show a range of differentiation states defined by phenotype (Table 2), function, anatomic localization and contribution to protection from reinfection. How and when is it decided which pathogen-specific CD8 T cells die after clearance of the pathogen (terminal effectors, TE) and which become long-lived memory is a question that is being vigorously studied and actively debated. In the context of this question, we will next discuss our current knowledge of memory CD8 T-cell differentiation as occurs following an acute infection.

Table 1. Defining characteristics of memory T cells

- Memory cells exhibit faster responsiveness upon encountering antigen. Memory T cells are more efficient than naïve cells in mounting an effector response.
- Besides the lymphoid compartment, memory T cells also localize in nonlymphoid and mucosal sites and can immediately confront the invading pathogen.
- Memory T cells are typically found at greater than naïve frequencies.

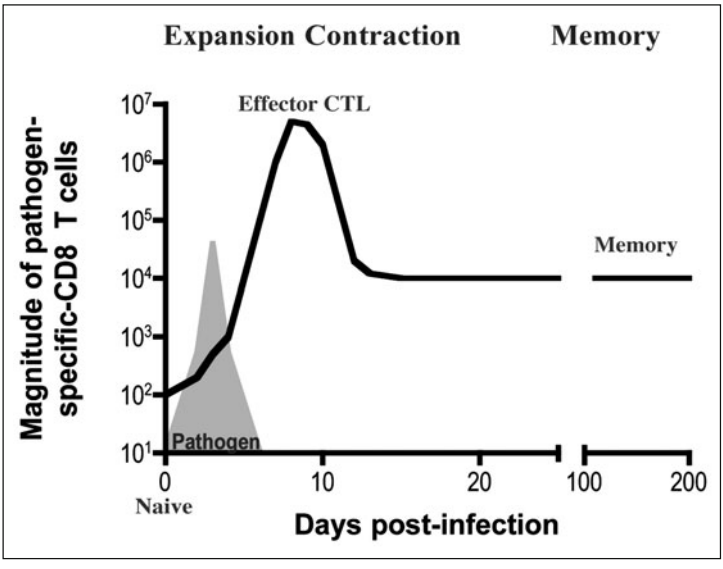

Figure 1. Antiviral CD8 T-cell responses. Illustration of the kinetics of CD8 T-cell response following infection of a mouse with a virulent pathogen such as *Listeria monocytegenes,* LCMV, VSV or vaccinia. Antigen-specific T cells clonally expand during the first expansion phase in the presence of antigen. After the virus is cleared the contraction phase ensues and the number of antigen-specific T cells decreases due to apoptosis. After the contraction phase the number of virus-specific T cells stabilizes and can be maintained for long periods (memory phase).

Programming during the Expansion Phase

CD8 T-cell responses are initiated when a naïve cell encounters antigen. In vivo, T-cell activation involves the transmission of two distinct inductive signals from APCs to naïve precursors. Signal 1 is antigen-specific and delivered *via* stimulation of the T-cell antigen receptor (TCR) by peptide-MHC class I complexes on the APC surface. Signal 2 (CD28) is costimulatory and serves to amplify or modify signal 1 by lowering the threshold required for responsiveness. A common theme that has emerged over recent years is that of "programming" of T-cell responses.[7] Several studies demonstrate that following initial antigenic instruction, the ensuing CD8 T-cell proliferation and effector cell differentiation events occur in a programmed fashion without further need for antigen.[8-12] Moreover, the onset and kinetics of contraction and memory differentiation are also programmed during the early stages of an immune response.[8,13] While these studies underscore the importance of instructive priming events in T-cell expansion, effector differentiation, contraction and memory generation, it is important to bear in mind that during an acute infection, other environmental cues are also collectively and progressively integrated during the course of CD8 T-cell immune responses to modulate CD8 T-cell differentiation.[14]

In addition to TCR and CD28 signals, recent data implicate the participation of a third signal in promoting strong CD8 T-cell expansion, development of effector functions and survival of the effector cells in vivo. This includes adjuvants, IL-12,[15-20] or Type-I interferons.[21,22] Using Type-I interferon receptor deficient CD8 T cells, it was observed that in the absence of direct Type-I interferon signals there was 99% reduction in CD8 T-cell expansion and memory generation. In recent years, it has been shown that CD8 T-cell expansion and cytotoxicity are also impaired in the absence of IL-21 signals.[23] Whether IL-21 signals can replace the third signal provided by IL-12 or Type-I interferons, or if IL-21 (and possibly another cytokine) is needed as an independent (fourth?) signal remains to be determined. Other factors that are dynamically regulated during an acute infection and have been implicated in impacting CD8 T-cell differentiation include

Table 2. Markers that distinguish between naïve, effector and memory T cells

	Marker	Naïve	Effector	Memory
I				
	CD44	Low	High	High
	CD11a	Low	High	High
	Ly-6C	Low	High	High
	CD122	Low	High	High
	CD123	Low	High	High
II				
	CD69	Low	High	Low
	CD25	Low	High	Low
	CD43	Low	High	Low
	KLRG-1	Low	High	Low
	Granzyme B	Low	High	Low
III				
	CD62L	High	Low	High
	CD127	High	Low	High
	CCR7	High	Low	High
	Bcl-2	High	Low	High
	CD27	High	Low	High
IV				
	IFN-γ	Low	High	High
	TNF-α	High	High	High
	IL-2	High	Low	High

This is a representative (not comprehensive) list illustrating the major patterns of changes seen as naïve T cells differentiate through effector and memory stages.

the strength and duration of antigenic stimulation, type of costimulatory signal (CD40, CD30, CD27, OX-40, 4-1BB, inducible costimulatory molecule ICOS), the complex cytokine milieu of inflammatory cytokines and growth factors, the type of antigen-presenting cells and interaction with other cell-types like CD4 T cells.[24-26] Signals through the inhibitory receptors (cytotoxic T-lymphocyte antigen 4 CTLA-4, B and T-lymphocyte attenuator BTLA and programmed death 1 PD-1, killer lectin-like receptor KLRG-1, 2B4, etc.) are also proposed to act to control the extent of expansion and effector differentiation and prevent immunopathology by blunting the immune response.[5,6,14]

Thus, collective assimilation of these signals directs the acquisition of key effector properties such as production of antiviral cytokines (IFN-γ, TNF-α), downregulation of lymphoid homing molecules (CD62L, CCR7) to enable peripheral tissue migration and cytolysis of infected target cells. This generation of potent CTLs is responsible for efficient pathogen clearance via direct effects on the pathogen and cytolysis of infected target cells.

Selective Survival of Memory Precursors during the Contraction Phase

After elimination of the pathogen, 90-95% of pathogen-specific CTL die by apoptosis during the contraction phase. Apoptosis primarily occurs by Fas and Bim pathways,[27] leaving behind a pool of surviving pathogen-specific cells that differentiate into long-lived memory cells. Towards distinguishing pathogen-specific CD8 T cells that are destined to die (terminal effectors, TE) from those that will live long-term to comprise the memory pool (memory precursors, MP), several studies were conducted. Based on these, we now know that the pathogen-specific CTL population is heterogenous and is marked by differential expression of various cell surface (CD62L, IL-7Rα, KLRG-1) and intracellular (IL-2, serine protease inhibitor 6, Spi6) markers.[28-31] Furthermore, differential expression of these markers is associated with diverse cell fates, in certain cases. For example, in acute infections, higher level of expression of IL-7Rα, Spi6 and IL-2 and lower expression of KLRG-1 by a subset of effector CD8 T cells correlates with selective survival and differentiation of this subset into long-lived memory cells.[32-36] Such phenotypic distinction of effector CD8 T cells into two subsets: one that preferentially survives following antigen clearance (memory precursors) and one that will predominantly die (terminal effectors) has opened up new lines of experimental pursuit to dissect the signals that drive their selective generation. Moreover, this observation suggests that the ability to survive during the contraction phase and differentiate into long-lived memory cells is actively programmed during the priming and expansion phase, whereby enhanced survival potential does not result from a passive, stochastic survival of a sub-population of effectors due to limitation of growth factors in the face of largely increased T-cell numbers.

However, little is known about the cell intrinsic and cell extrinsic mechanisms that control contraction. While IL-2 and Spi6 expression directly correlates with memory precursors, their precise role in mediating selective survival of memory precursors during the contraction phase is unclear. It is proposed that by inhibiting granzyme activity, Spi6 protects effector CTL from damage during target cell killing. The availability of growth factors including IL-15, IL-7 and IL-2 have been proposed to play a crucial role during the contraction phase and memory T-cell maintenance.[37-39] Rapid contraction is indeed observed in the absence of IL-15 signals, but the size of the resultant memory pool in IL-15-/- mice is similar to that in wild-type mice.[40,41] This suggests that selective survival of memory precursors is independent of IL-15 signals. While higher IL-7Rα effectively distinguishes memory precursors during an acute infection, studies involving augmented delivery of IL-7 signals did not lead to enhanced memory generation,[42-45] suggesting that IL-7 signals are not sufficient to drive the preferential survival of memory precursors. Additionally, studies showing similar contraction in the setting of an acute infection where CD8 T cells lacked IL-2Rα expression[45,46] are suggestive of IL-2 independent survival of memory precursors during the contraction phase. Knowledge of various cell intrinsic and extrinsic factors that regulate contraction will aid in the manipulation of the kinetics and quantity of CD8 T-cell memory.

Memory CD8 T-Cell Differentiation and Heterogeneity

After antigen is cleared, terminal effector cells are eliminated during contraction, leaving behind memory precursors that give rise to the long-lived memory pool. Differentiation of memory CD8 T cells is a progressive process wherein key genotypic, phenotypic and functional properties are acquired over several weeks following antigen clearance.[47-49] In the absence of antigen, virus-specific CD8 T cells return to a resting phenotype by downregulating the expression of certain effector molecules such as granzyme B, while progressively acquiring key memory properties of rapid proliferation upon exposure to antigen and antigen independent homeostatic proliferation in response to IL-7 and IL-15 cytokines. It is noteworthy that not all effector functions are downregulated during transition of effector cells into memory; memory CD8 T cells retain the ability to rapidly produce IFN-γ and TNF-α upon reexposure to antigen. Memory cells can also quickly reacquire cytotoxic activity upon secondary antigen encounter. Combined with elevated frequencies of virus-specific memory cells compared to naïve cells (upto 1000-fold higher frequencies), the ability to rapidly mount effector functions renders memory cells more efficacious than naïve CD8 T cells at combating infection (Table 1).

Processes involved in conversion from effector to memory stage are largely unknown. Two recent studies in Nature by Araki et al. and Pearce et al[49a, 49b], demonstrate for the first time that metabolic changes in T cells may be crucial for T-cell memory generation. Using the immunosuppressive drug, rapamycin, which inhibits mTOR signaling, Araki et al.[49a] showed that treating mice with rapamycin during the expansion phase led to enhanced generation of memory precursors and long-lived memory T cells. Furthermore, treatment during the contraction phase sped up the conversion of effector T cells to long-lived memory cells with superior recall ability. It was also shown that mTOR functioned in a T cell intrinsic manner to regulate memory cell differentiation. In a parallel study, Pearce et al[49b], found compromised memory generation in TRAF-6 deficient mice. In microarray analyses they found that in the absence of TRAF-6, which is a negative regulator of T-cell signaling, several metabolic pathways such as fatty acid oxidation were defective. Compromised memory T-cell generation in TRAF-6 deficient mice could be reversed by treatment with anti-diabetic drug metformin, or by rapamycin. Both these drugs affect cellular metabolism; while metformin activates AMP kinase, an enzyme that inhibits mTOR activity, rapamycin directly inhibits mTOR. Typically, mTOR is activated by antigen-induced TCR signaling and growth factors, and regulates various cellular processes including cell growth and metabolism, autophagy, etc. While these studies suggest that an alteration of metabolic state via mTOR inhibition may be crucial for effector to memory conversion, how a change in metabolic state of a T cell could enhance memory T-cell numbers and function remains to be determined. Moreover, manipulation of mTOR and key downstream signaling molecules holds promise for improving future vaccine strategies.

The memory CD8 T-cell compartment is characterized by significant heterogeneity with respect to surface protein expression, gene expression, effector functions, proliferative potential and contribution to protection from reinfection and trafficking. Two main cell-types involved in CD8 T-cell memory are effector memory (T_{EM}) and (CD62L-/CCR7-) central memory (T_{CM}) (CD62L+/CCR7+) cells.[48,50,51] T_{CM} cells are concentrated in secondary lymphoid tissues and have little or no immediate effector functions. Instead, they respond to antigen by rapidly dividing and differentiating into effector cells. Moreover, they possess stem cell like qualities of self-renewal in response to homeostatic cytokines including IL-7 and IL-15. T_{EM} cells, on the other hand, can migrate to peripheral tissues and mount a more pronounced immediate cytolytic activity compared to T_{CM} cells. T_{EM} cells undergo modest proliferation upon antigenic stimulation, albeit to lower levels than T_{CM} cells. Together, both T_{EM} and T_{CM} cells contribute to protective immunity depending on the nature and route of infection. Besides this well-defined T_{EM}/T_{CM} dichotomy of recirculating memory CD8 T cells, additional levels of complexity in memory CD8 T-cell phenotypes exist between distinct peripheral tissues and in different infectious models; for example, pathogen-specific lymphocytes residing in the gut, lung-airways or brain retain a distinguishing CD69 expression.[6] Such functional, anatomic and phenotypic heterogeneity in the CD8 T-cell memory pool has important consequences for immunity and the factors that govern this cell fate decision are of major interest.

Molecular Basis of Optimal Memory Functions

Accelerated, more efficacious recall responses of memory cells result from a reprogramming of gene expression profile by epigenetic changes involving DNA methylation, histone modifications and reorganization of chromatin structure.[52,53] Moreover, accelerated demethylation of the IFN-γ promoter by a putative enzymatic factor specifically active in memory cells may present an additional novel mechanism of differential gene expression.[54] In addition to epigenetic changes, heritable programs of gene expression are also maintained by continued expression of certain transcription factors such as the tissue-specific T-box transcription factors T-bet and eomesodermin.[55,56] Interestingly, mice with mutations of the genes encoding T-bet and eomesodermin exhibit defective effector cytotoxic programming, decreased expression of CD122 and are nearly devoid of IL-15 dependent memory CD8 T cells. These studies provide a molecular link between programming of effector and memory CD8 T cells and exemplify a framework in

which transcription factors specifying lineage function can also specify responsiveness to growth signals. In addition, transcriptional repressor BCL6b has been shown to enhance magnitude of secondary response of memory CD8 T cells independent of primary responses.[57] At a molecular level rapid proliferative responses of memory upon rechallenge are also attributed to modifications in TCR signal transduction machinery leading to more sensitive and rapid assimilation of stimulatory signals.[58] Moreover, memory cells are precharged with several factors necessary for G1- to –S-phase transitions,[59,60] thereby suggesting that they may require a lower threshold of stimulation to enter cell cycle.

Models of Memory CD8 T-Cell Differentiation

The differentiation path followed by memory CD8 T cells is keenly studied. Whether memory cells arise as direct descendants of effector cells (linear differentiation model), or develop as a separate lineage from naïve cells (divergent differentiation model) has long been debated (Fig. 2). Technological advances are now making it possible to distinguish between these two possibilities

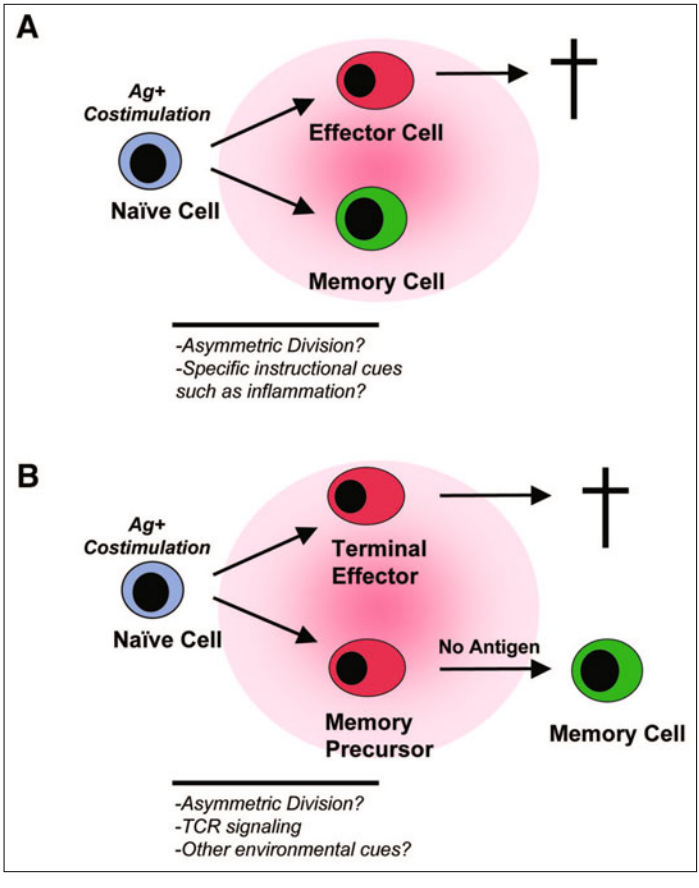

Figure 2. Models of memory cell differentiation. A simplistic illustration of the currently debated models of T-cell differentiation is presented. Model 1 represents the B-cell paradigm of divergent pathways followed by effector and memory T cells, such that following activation of naïve cells, divergent effector and memory lineages are generated via asymmetric division. Model 2 is a representation of the more conventional linear pathway of differentiation of naïve T cells into effector cells and ultimately memory cells.

more incisively, yet data supporting both models of memory T-cell generation exist. The conventional model of memory CD8 T-cell differentiation is the linear differentiation model, which proposes that memory cells are derived directly from effector cells. In fact, several studies have shown that T-cell activation and proliferation are tightly coupled to effector cell and eventually memory cell differentiation.[47,61] The use of CRE/LOXP system in transgenic mice to indelibly 'mark' (via Cre-mediated recombination) pathogen-specific effector T cells that have activated a "signature" effector gene (granzyme B) promoter with a reporter molecule (alkaline phosphatase or a fluorescent molecule)[62,63] showed that 'marked' effectors were maintained in the memory T-cell pool. Using this elegant system, it was found that effector cells that upregulated granzyme B expression form long-lived memory cells in both lymphoid and nonlymphoid compartments, indicating that both T_{CM} and T_{EM} cells are direct descendants of effector cells. However, this experimental system cannot distinguish between TE and MP cells and does not allow one to ask the question whether intrinsic differences between TE and MP cells, other than granzyme B expression, may be responsible for their diverse cell fates. With the ability to now phenotypically distinguish TE and MP cells, detailed protein expression, gene profiling and functional analyses of these subsets has demonstrated that memory precursors are remarkably similar to terminal effectors in their effector differentiation.[35] This further supports the paradigm that memory T cells pass through an effector phase. However, whether transition through an effector stage is obligatory for memory generation is unclear from these studies.

The second model of memory differentiation proposes that memory T-cell development occurs in a nonlinear fashion without passing through a fully functional effector phase. Thus, asymmetric division after activation of a naïve T cell can lead to the formation of two distinct daughter cells with polarized terminal effector and memory cell fates, due to unequal partitioning of proteins during the first division.[64] In certain cases (for e.g., activation with heat killed bacteria, or in vitro stimulation with high doses of IL-2 or IL-15 cytokines)[65,66] memory T cells have been shown to develop without passing through an effector-cell stage. Depending on the priming milieu, it is proposed that antigen plus costimulation in the presence of an inflammatory milieu early during an infection (for e.g., IL-12, Type-I interferons and IL-21 signals) may favor differentiation of effector T cells, whereas antigen plus costimulation in the absence of inflammation (as antigen and infection are waning) may lead to memory T-cell differentiation.[36,65-71] A recent study provides evidence that the quality of TCR signals can also determine effector versus memory development.[72,73] By introducing point mutations in the transmembrane domain of TCR-β, which leads to poor polarization of the TCR to the immunological synapse without any evident effects on T-cell-APC interaction in vitro, the investigators found that the effector differentiation was unaltered, but pathogen-specific memory pool was largely abrogated. While this study clearly demonstrates that the quality of signal 1 from the TCR can direct transcriptional programs that are unique to effector versus memory development, it does not provide incontrovertible proof for the divergent model, as it is unclear whether memory precursors were generated in this system but failed to differentiate into functional memory cells. Also, whether the signaling defects that are associated with lack of memory generation in this study are recapitulated in vivo is unclear at present.

In summary, presently evidence in support of both linear and divergent models of memory differentiation exist and additional creative approaches are needed to resolve this issue. The key to understanding the differentiation path followed by memory T cells will be to identify true memory precursors at the earliest possible time during an immune response and to determine the signals required for their generation.

Mechanisms Regulating Memory CD8 T-Cell Heterogeneity

While developing complete models of memory T-cell differentiation, it is also important to consider the heterogeneous nature of memory T-cell pool. What is the source of memory T-cell heterogeneity? Is this continuum of differentiation states and/or lineages programmed via unique transcriptional regulation that is cell autonomous and can cell extrinsic factors be manipulated to dictate the final outcome of the differentiation process? As discussed above, early priming events strongly influence the number, location and functional properties (quality) of memory CD8 T cells.

Although, antigen exposure is needed only briefly (20-24 hrs) to initiate T-cell development, the type of effector and memory CD8 T-cell responses eventually generated is further influenced by the duration and/or dose and the "context" of antigenic stimulation (for e.g., cytokine milieu, chemokines signals and costimulation, as determined by the nature and activation state of APCs). Distinct lymphoid environments have also been shown to program T cells to adopt different trafficking properties, thereby implicating unique environmental cues in possibly dictating memory outcome. Additionally, following emigration from secondary lymphoid tissue, inductive signals unique to distinct anatomical compartments may further regulate memory CD8 T-cell differentiation by providing a unique milieu of cytokines, costimulation, immune accessory cells and antigen persistence.

It is believed that the balance between effector and memory cells and the heterogeneity in memory population is directly related to the extent and frequency of TCR stimulation[5,6,50,67,74-77] and the division history of the cells (likely conditioned by the dose of the antigen), such that functionally fit memory cells arise only under optimal stimulation conditions in which antigen load is effectively controlled. This is incorporated in the decreasing potential model of memory differentiation (Fig. 3, which proposes that the potential of effector CD8 T cells to differentiate into memory cells is

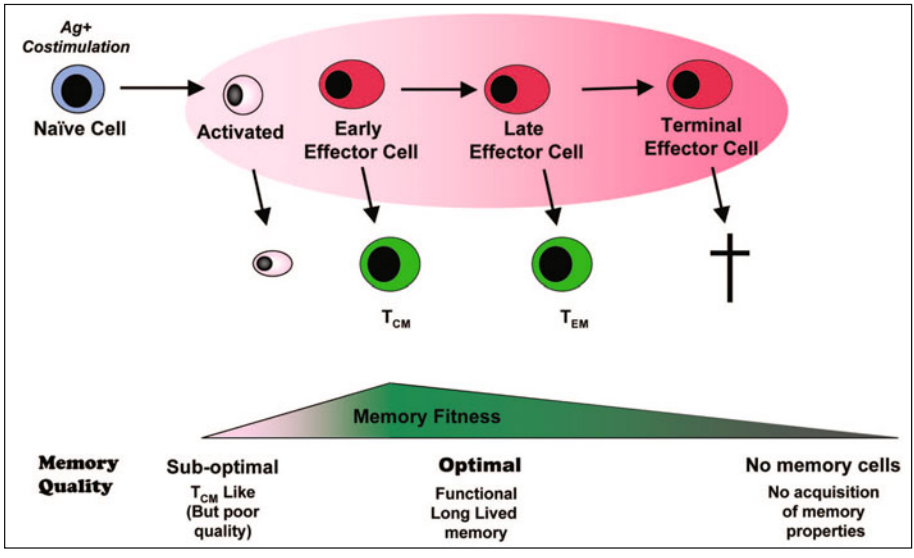

Figure 3. The decreasing potential model of memory CD8 T-cell development. Optimal antigenic stimulation triggers a developmental program of expansion and differentiation of naïve T cells into effectors, a fraction (5-10%) of which progressively differentiate into potent long-lived memory cells following antigen clearance. Whereas suboptimal stimulation may lead to limited CD8 T-cell expansion and/or impaired memory development and function, prolonged antigenic stimulation also impairs memory generation potential by driving the cells towards a terminally differentiated effector phenotype. Thus, the decreasing potential model postulates that cells become progressively terminally differentiated with increasing stimulation and cell division. This is accompanied by an increasing susceptibility to apoptosis and cells receiving the highest magnitude of stimulation possess the lowest potential to survive and differentiate into memory cells. Furthermore, the generation of lymphoid and nonlymphoid lineages and the rate of development of lymphoid memory cells are also regulated by the duration and/or strength of antigenic stimulation. Whereas a short duration of antigenic stimulation favors the development of T_{CM}, longer stimulation favors the differentiation of terminal T_{EM} cells. Apart from antigen, additional cell-extrinsic variables including the cytokine and chemokine milieu, costimulatory and inhibitory signals (dependent on the type and activation state of the APC), interaction with other cell-types (for e.g., CD4 T cells) and the anatomic location might further impact the qualitative and quantitative aspects of a developing T-cell response and the ensuing memory differentiation and maintenance.

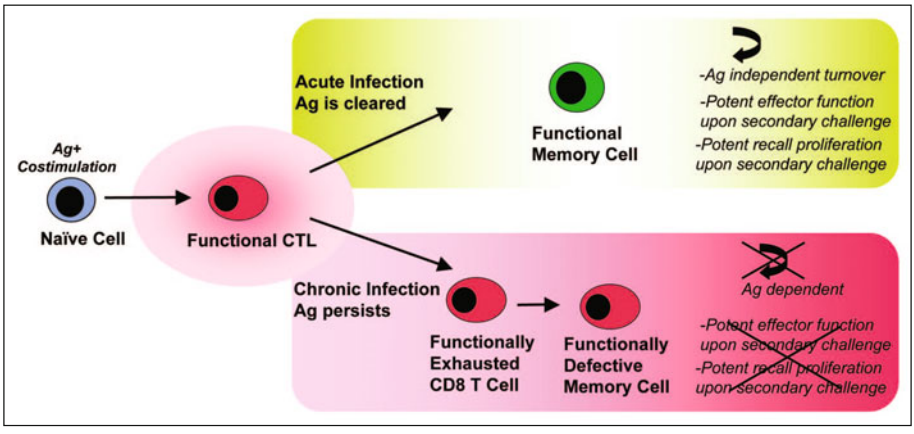

Figure 4. CD8 T-cell differentiation during acute and chronic infections. Acute viral infections are characterized by clearance of virus and progressive differentiation of CD8 T cells into functional memory cells capable of IL-7 and IL-15 driven homeostatic proliferation in the absence of antigen and robust recall proliferation and effector responses upon secondary encounter with the pathogen. Although early events during acute and chronic infections are presumed to be similar, antigen persistence during chronic infections results in altered differentiation of virus-specific CD8 T cells. Functional exhaustion develops as early as the effector phase with progressive loss of functions. Memory T cells are also functionally defective when pathogen persists and are unable to homeostatically maintain their numbers in the absence of antigen.

progressively lost with increased antigenic stimulation.)[74] Several studies have helped further refine this model to explain the generation of memory T-cell heterogeneity. It is proposed that whereas, suboptimal stimulation might lead to limited T-cell expansion and memory development, optimal TCR signal integration during activation of naïve cells leads to the generation of effector cells that have the potential to differentiate into memory cells.[6] Whereas a short duration of antigenic stimulation favors T_{CM} generation, longer stimulation promotes the generation of T_{EM} and TE cells thereafter. In support of this, reducing antigen load, by antibiotics or by using higher CD8 T-cell precursor frequencies, was found to lead to a more rapid conversion to T_{CM} phenotype (CD62L+).[30,48,78-84] Since the expression pattern of CD62L on activated T cells appears to be regulated by antigenic stimulation (initial TCR stimulation results in rapid shedding of CD62L from cell-surface by proteolytic cleavage, but continued TCR stimulation leads to transcriptional silencing of CD62L encoding locus), it is interesting to speculate that differential modes of CD62L downregulation may relate to differential reexpression during memory development. The decreasing potential model of memory differentiation also explains CD8 T-cell dysfunction in chronic infections where antigen persists (Fig. 4). In this case effector T cells do not differentiate into durable memory cells and may survive in an antigen-dependent manner as dysfunctional cells (exhaustion), or may eventually die (deletion). The degree to which CD8 T cells become defective appears to correlate with antigen load and can range from partial loss of cytokine production to complete loss of cytolytic function and cytokine secretion as discussed below.

CD8 T-Cell Responses following Persistent Infection

CD8 T-cell differentiation pathway described above represents the paradigm for most acute infections. However, under conditions of chronic infections where antigen persists several aspects of a normal CD8 T-cell response are altered (Table 3).[85] First, the hierarchy of epitope-specific CD8 T cells may be skewed such that subdominant specificities can predominate the virus-specific T-cell response, while immunodominant specificities may even be lost in certain cases.[86,87] Second,

Table 3. Comparison of CD8 T-cell differentiation in acute and chronic infections

	Characteristic	CD8 T-Cell Differentiation
Acute Infection		
	Virus is cleared and T cells experience rest from antigen	Functional memory CD8 T cells capable of antigen-indepent longterm persistence develop
Persistent Infection		
	Persisting high level viral replication leads to continuous TCR stimulation, such that there is no rest from antigen	Functional exhaustion (loss of cytokine production and CTL activity) and deletion of certain CD8 T-cell clones occurs. Exhaustion correlates with expression of inhibitory cell surface receptors. Memory cells are unable to persist in the absence of antigen

the tissue distribution of virus-specific CD8 T cells may be altered, such that virus-specific cells preferentially localize in nonlymphoid tissues. This is likely driven by antigen localized in these compartments or by altered expression of homing molecules on virus-specific CD8 T cells. Third, chronic infections result in severely impaired T-cell function (functional exhaustion) and can also lead to physical elimination of responding T cells (deletion). Fourth, the molecular requirements for maintenance of virus-specific CD8 T cells during chronic infections are also altered.[88] In the following sections, we will describe the altered CD8 T-cell responses observed during chronic infections and discuss our current understanding of the molecular basis of CD8 T-cell dysfunction.

Functional Exhaustion of CD8 T Cells during Chronic Infections

One of the key properties of memory CD8 T cells generated following acute infection is that they maintain the ability to reactivate antiviral effector functions upon antigenic stimulation. Exhaustion or loss of effector function was first reported in mice chronically infected with LCMV.[86] During chronic LCMV infection, there is a hierarchical loss of the ability to perform effector functions, starting during the effector phase and becoming progressively more severe as the infection progresses.[89] This exhaustion of effector functions occurs in a hierarchical manner. First, properties such as the ability to produce IL-2 and mount cytolysis and robust proliferation are lost at early stages of exhaustion. During this stage there may be loss of TNF-α production as well, which appears to be more resistant to exhaustion than IL-2. As infection progresses, IFN-γ production also begins to be compromised, ultimately leading to functionally inactive virus-specific cells that do not produce IL-2, TNF-α or IFN-γ and are incapable of ex vivo cytotoxicity. If antigen load in the form of MHC/peptide complexes presented in vivo is high, epitope-specific CD8 T cells can be physically deleted. During chronic LCMV infection, this is the case for two immunodomiant responses (Db/NP396 and Kb/GP34).[87] This continuum of inactivation, with loss of function becoming progressively worse as either viral load or the duration of infection increases, is distinct from T-cell anergy, wherein acquisition of function is impaired to begin with following priming. Functional exhaustion is not limited to chronic LCMV infection, but is also observed in other animal models such as polyoma virus,[90] Friend's leukemia virus,[91] adenovirus,[92] mouse hepatitis virus[93] and SIV infection of macaques.[94]

Altered Memory CD8 T-Cell Homeostasis

A hallmark of memory CD8 T cells generated following acute infections is their ability to persist in the absence of antigen. Longevity of memory T cells, perhaps indefinite, is attributed to their stem cell-like quality of replenishing their numbers in the absence of antigen via homeostatic proliferation. This property of self-renewal in the absence of antigen distinguishes memory CD8

Table 4. T-cell homeostasis in acute and chronic infections

- Memory CD8 T Cells can persist and maintain their numbers in an antigen-free environment by undergoing homeostatic proliferation to replenish their pool. Homeostatic proliferation occurs in response to IL-15 and IL-7 and does not require stimulation with cognate antigen.
- In chronic infections many pathogens can persist and provide continuous or intermittent stimulus to maintain memory T-cell numbers.

T cells from naïve and effector cells (Table 4). Clearly factors that enhance cell division (IL-15) or promote cell survival (IL-7) are important in maintaining the numbers of memory T cells in the absence of antigen. Bone marrow is the preferential homing site for memory T cells,[95,96] where they proliferate more extensively than in secondary lymphoid organs in response to self-renewal signals, which are likely produced constitutively by specific cell-types within the bone marrow.

Contrary to acute infections, virus-specific CD8 T cells generated during chronic LCMV infection fail to persist when adoptively transferred into naïve mice.[88,89,97-99] This defect in CD8 T-cell homeostasis correlates with decreased expression of CD127 and CD122, the receptors for homeostatic cytokines IL-7 and IL-15. Similar observations of loss in homeostatic proliferation in response to IL-15 was also reported for CD8 T cells generated in response to the murine γ-herpesvirus infection of mice[100] and is likely not limited to mouse models of chronic viral infection. Although virus-specific CD8 T cells from chronically infected mice respond poorly to IL-7 and IL-15, they are maintained for long-periods in chronically infected mice. This maintenance is apparently dependent on the presence of infection since virus-specific cells decline when adoptively transferred into uninfected hosts. As opposed to the slow and steady homeostatic proliferation of antigen-independent memory cells generated following acute infections, CD8 T cells are maintained in chronic infections via extensive proliferation.[99] This suggests an altered homeostatic regulation in persistent infections.

Mechanisms of CD8 T-Cell Exhaustion

As we continue to understand the underlying molecular causes of CD8 T-cell exhaustion, it is important to note that the functional programming of memory responses during persistent infection of mice is not hardwired during priming but is alterable and is impacted by continuous instruction from the antigenic environment. Through an elegant set of adoptive transfer experiments,[101] it has been shown that removal of dysfunctional T cells from the infection and/or antigenic milieu bears the potential to rescue T-cell functionality. When dysfunctional CD8 T cells are transferred from a persistently infected mouse into a mouse that has cleared an acute infection, reversal of T-cell dysfunction is observed such that cells regain their ability to produce TNF-α and IL-2 and also upregulate the expression of survival molecules CD127 and Bcl-2. However, restoration of function was dependent on the extent of CD8 T-cell dysfunction, such that longer duration of persistent infection resulted in a progressive loss of functional recovery potential. This provides a basis for future therapeutic strategies to treat persistent viral infections.

Understanding the molecular basis of CD8 T-cell exhaustion is an area of intense research. CD8 T-cell exhaustion is marked by gene expression changes, such that the transcriptional profile of exhausted CD8 T cells differs from that of naïve CD8 T cells as well as functional effector and memory cells generated during an acute infection.[102] Interestingly, exhausted CD8 T cells generated following chronic LCMV infection exhibit overexpression of several inhibitory receptors (PD-1, 2B4, CTLA-4, LAG-3, CD160, etc), which in certain cases is even nonredundant.[103] Using the mouse model of chronic LCMV infection, programmed death-1 (PD-1), an inhibitory receptor in the CD28 superfamily, has been found to serve as an important negative regulator of T-cell function.[104] Exhausted CD8 T cells express high levels of PD-1 compared to functional memory cells and blockade of PD-1/PD-L1 interactions results in enhanced T-cell function and viral control. HIV-1 and HCV-specific CD8 T cells also express high levels of PD-1 and in

the case of HIV-1 patients, the level of PD-1 expression correlates directly with viral loads and inversely with CD4 T-cell counts.[105-110] Furthermore, HIV-1 long-term nonprogressors expressed lower levels of PD-1 than progressors and PD-1 expression declined in viremic patients following initiation of HAART.[108,111] In vitro blockade of the PD-1/PD-L1 pathway on human cells led to enhanced proliferation and improved function of HIV-specific CD4 and CD8 T cells as well as HCV-specific CD8 T cells.[105-110] Whether different infections upregulate unique inhibitory receptors and whether different inhibitory receptors act cooperatively to downregulate T-cell responses in chronic infections are important questions that will guide development of therapeutic approaches specific to a particular pathogen. Additionally, the mechanisms by which CD8 T-cell function is restored by blockade of inhibitory receptors present another important question that will further our understanding of CD8 T-cell exhaustion.

IL-10 has also been recently implicated in limiting optimal T-cell responses during chronic infections.[112-114] Mice lacking IL-10 or blockade of IL-10R led to efficient control of replication of chronic LCMV and development of functional T-cell responses.[112,113] In contrast, control mice progressed to chronic infection. These results suggest that the IL-10/IL-10R pathway plays a key role in early events that determine whether an infection is rapidly cleared or becomes chronic with T-cell dysfunction. Additionally, Foxp3+ regulatory T cells (Tregs) can also influence the quality and potency of antiviral CD8 T cells directly by modulating CTL function and indirectly via production of immunoregulatory cytokines or inhibition of APC maturation. Given the potent ability of Tregs to suppress T-cell proliferation in vitro[115,116] and their role in modulating the cytotoxicity of CD8 T cells in vivo,[117] it is possible that different negative regulatory pathways such as IL-10, PD-1 and Tregs may regulate different effector T-cell properties during chronic infection.

Conclusion

In conclusion, recent years have seen major advances in the field of CD8 T-cell memory differentiation. With the molecular distinction of memory precursors and terminal effectors, we are now uniquely poised to ask important mechanistic questions pertaining to the generation of memory cells. For example, precisely when during an immune response are memory cells generated? What are the signals that regulate the generation and developmental program of memory cells? Is there a unique transcriptional signature comprising memory-specific genes? What are the precise mechanisms regulating T-cell exhaustion? Can dysfunctional CD8 T cells be rescued at any stage of exhaustion? Answers to these and many other exciting questions will help move the field forward towards a more rational design of vaccines that aim at inducing potent CD8 T-cell immunological memory to chronic viral infections and cancer.

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Chapter 8

Longevity of T-Cell Memory following Acute Viral Infection

Joshua M. Walker and Mark K. Slifka*

Abstract

Investigation of T-cell-mediated immunity following acute viral infection represents an area of research with broad implications for both fundamental immunology research as well as vaccine development. Here, we review techniques that are used to assess T-cell memory including limiting dilution analysis, enzyme-linked immunospot (ELISPOT) assays, intracellular cytokine staining (ICCS) and peptide-MHC Class I tetramer staining. The durability of T-cell memory is explored in the context of several acute viral infections including vaccinia virus (VV), measles virus (MV) and yellow fever virus (YFV). Following acute infection, different virus-specific T-cell subpopulations exhibit distinct cytokine profiles and these profiles change over the course of infection. Differential regulation of the cytotoxic proteins, granzyme A, granzyme B and perforin are also observed in virus-specific T cells following infection. As a result of this work, we have gained a broader understanding of the kinetics and magnitude of antiviral T-cell immunity as well as new insight into the patterns of immunodominance and differential regulation of cytokines and cytotoxicity-associated molecules. This information may eventually lead to the generation of more effective vaccines that elicit T-cell memory with the optimal combination of functional characteristics required for providing protective immunity against infectious disease.

Introduction

The concept of immunological memory is well established, but it was not until the twentieth century that the cellular origins of antimicrobial immunity and the basis of immunological memory first began to be elucidated.[1] It is remarkable how the field of immunology has changed in the four decades that have passed since Mitchell and Miller postulated the existence of T cells as a distinct subset of small lymphocytes[2] (Fig. 1). During this time, several of the initial questions regarding T-cell specificity and function have been answered and yet many new questions regarding the dynamics and functional attributes of the memory T-cell compartment have been raised. To answer these questions, a multitude of quantitative techniques have been developed and optimized to assess T-cell memory. In the early days of immunology, these techniques often consisted of bulk analysis of broadly defined cell populations with little understanding of the mechanisms employed in their function. Today, it is possible to analyze cytokine production, cytolytic potential and the phenotype of highly defined subsets of even rare T-cell populations directly ex vivo. Thus, the evolution of T-cell analysis (Fig. 1) is characterized by a trend toward measuring more precisely defined T-cell subsets and developing a progressively more refined ability to determine function at the single cell level.

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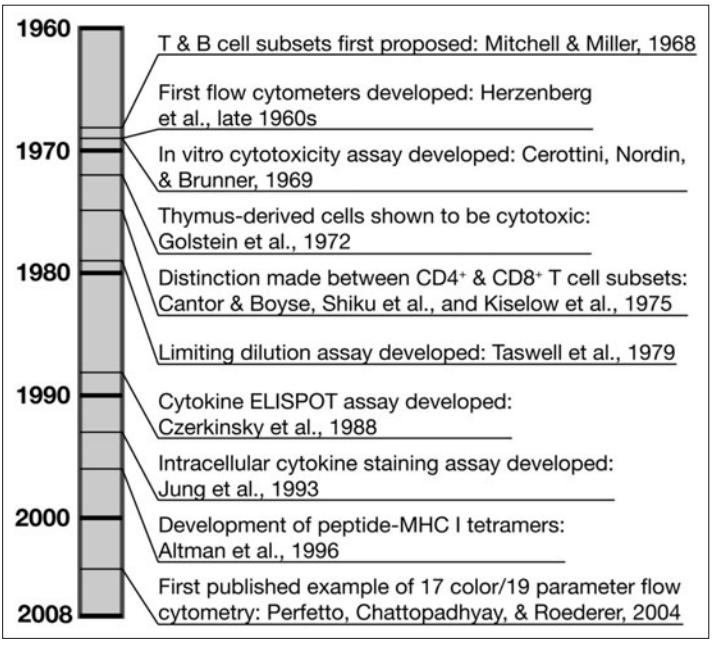

Figure 1. Historical advances involved with analysis of T-cell memory. In 1968, Mitchell and Miller proposed the existence of a subset of lymphocytes that were thymus-derived and distinct from bone marrow-derived B-cells,[2] setting the next four decades of T-cell research in motion. The development of the in vitro cytotoxicity assay in the late 1960s[3] made it possible to determine that T cells and not B cells, were the component of small lymphocytes that were directly cytotoxic.[4] In the mid 1970s, the concept of $CD4^+$ and $CD8^+$ T-cell subsets arose when it was determined that helper activity and cytotoxic activity were restricted to distinct subpopulations of T cells.[6,7] In the late 1960s, the first flow cytometers were developed[75] and this technology would later come to play an integral role in T-cell analysis. In the late 1970s, the limiting dilution assay was developed.[8-10] This is a significant landmark in T-cell analysis because the limiting dilution assay was the first technique to quantitatively assess T-cell responses to antigen. In the late 1980s, the cytokine ELISPOT (enzyme-linked immunospot) assay was developed, allowing T-cell responses to be quantitatively measured without requiring ex vivo T-cell expansion.[11] In the early 1990s, intracellular cytokine staining assays were developed, making it possible to quantitate the expression of multiple cytokines directly ex vivo.[12-14] In 1996, peptide-MHC Class I tetramers were developed.[15] The introduction of tetramer reagents made it possible to quantitatively measure the frequency of peptide-specific T-cell populations regardless of their function/cytokine profiles and without performing ex vivo restimulation. Since the antigen-specific T cells do not require restimulation to be visualized with peptide-MHC Class I tetramers, the native in vivo phenotype of the cells is also preserved. By the late 1990s and through today, polychromatic flow cytometry[25,75] has revolutionized the study of T-cell function and phenotype by making it possible to simultaneously analyze T-cell lineage markers, phenotype, cytokine profiles and cytotolytic protein expression of even rare T-cell populations directly ex vivo.

Cytotoxic activity has been a mainstay of T-cell analysis since its development in the 1970s. Through the use of Cr51-release assays, Cerottini et al demonstrated that the cytotoxic T cells present in a mixed population of lymphocytes could be depleted with anti-θ antibodies.[3] Shortly thereafter, others proved that depletion of B cells had no effect on the cytotoxic properties of mixed lymphocyte populations,[4] proving that it was indeed the thymus-derived lymphocyte population that was responsible for cell-mediated destruction of allogenic targets. This discovery

was followed by the work of two independent groups who demonstrated that cells bearing the CD8 antigen were responsible for the cytolytic activity of T cells,[5-7] further defining this population. During this time, Taswell and colleagues developed limiting dilution assays which allowed the frequency of antigen-specific T cells to be quantitatively determined.[8-10] Limiting dilution assays remained the cornerstone of T-cell quantitation until the late 1980s and early 1990s when the development of cytokine ELISPOT assays,[11] intracellular cytokine staining (ICCS)[12-14] and peptide-MHC Class I tetramers[15] greatly expanded the number of techniques that could be used for quantitatively measuring T-cell responses directly ex vivo. In a landmark study published in 1998, Murali-Krishna et al[16] demonstrated that IFNγ ELISPOT assays, IFNγ ICCS and peptide-MHC Class I tetramers all identified the same approximate frequency of peptide-specific $CD8^+$ T cells following infection of mice with lymphocytic choriomeningitis virus (LCMV). Moreover, they compared these new techniques to the standard approach of limiting dilution analysis and demonstrated that the number of antigen-specific T cells determined by the older technique was off by 10-fold or more. In other words, limiting dilution analysis, the best technique available up until the 1990's, was detecting <10% of the total virus-specific T-cell response identified by ELISPOT, ICCS, or peptide-MHC Class I tetramers. By performing T-cell quantitation by each of these approaches in one comprehensive study, this work provided the first "Rosetta stone" for understanding how these different approaches to T-cell quantitation compared with each other in direct side-by-side analysis. Remarkably, peak antiviral T-cell responses to LCMV reach about 50-75% of the total $CD8^+$ T cells in the spleen.[16,17] This is likely due to the tropism of the virus; LCMV infects lymphoid tissues such as the spleen and virus-specific T cells preferentially home to, and proliferate at, sites of infection. Peak $CD8^+$ T-cell responses against vaccinia also reach ~25% of total splenic T cells[18] and frequencies of virus-specific T cells in nonlymphoid organs can also be quite high.[19] For instance, the frequency of virus-specific T cells may reach 25-80% of the total T-cell population in the lungs following acute respiratory infection[20-22] or in the brain following infection with neurotropic viruses.[23,24] The high frequency of virus-specific T cells observed in these murine studies was thought to be restricted to rodent models of acute viral infection. However, as discussed later in this chapter, high frequencies of virus-specific T cells are now being identified during acute human infection as well. With the advent of polychromatic flow cytometry, in the last decade it has become possible to analyze ten or more fluorescence parameters[25] and this now makes it feasible to simultaneously analyze phenotype, cytokine production, cytolytic potential, proliferative status and viability of T cells identified by peptide-MHC tetramers or by antigenic stimulation. These advances in technology have lead to exciting new developments in our understanding of human T-cell memory.

Memory T-Cell Responses following Acute Viral Infection

T-cell memory can be surprisingly long-lived and studies examining the duration of cellular immunity following smallpox vaccination have demonstrated that antiviral $CD4^+$ and $CD8^+$ T-cell responses can be identified for up to 75 years after a single acute viral infection.[26,27] Measurement of virus-specific T-cell frequency however, represents only one dimension of T-cell memory and as our ability to quantitate and functionally assess T cells has evolved, our understanding of the dynamics and duration of T-cell memory to acute viral pathogens has continued to grow. There are no cross-reactive orthopoxviruses endemic to the US and so analysis of immunity following smallpox vaccination with vaccinia virus (VV) provides an opportunity to measure T-cell memory in the absence of environmental re-exposure.[27] Likewise, analysis of T-cell memory following childhood measles virus (MV) infection or vaccination (e.g., MMR; measles, mumps, rubella vaccination) or yellow fever virus (YFV) vaccination also provide important information regarding T-cell memory to viruses that cause only rare outbreaks (MV) or are no longer endemic in the US (YFV).

Edward Jenner was the first to formally demonstrate long-term protective immunity against orthopoxviruses[28] and in 1800, he published a report demonstrating that immunity following cowpox infection (the basis of contemporary smallpox vaccination) could be maintained for >50 years after infection.[29] Over 200 years passed before the technology was available to quantitatively

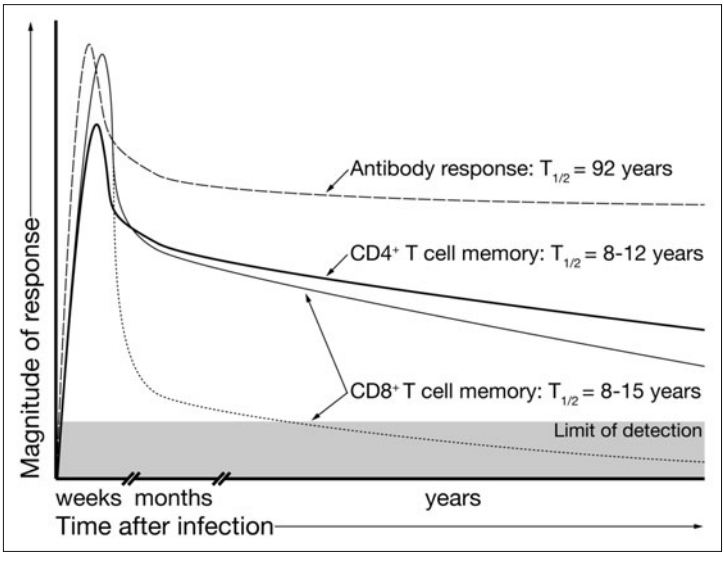

Figure 2. Immunological memory following vaccinia virus (VV) infection. Vaccinia virus infection provides a prototype for understanding the kinetics and duration of antiviral immunity following acute viral infection. Antiviral antibody responses peak within a few weeks after infection and after passing through a short period of decline, the humoral immune response remains quite stable, declining with a half-life of approximately 92 years.[76] Virus-specific $CD4^+$ and $CD8^+$ T-cell responses peak within 2-3 weeks after infection[31-35] and then following an early rapid decay rate, the estimated half-life of T-cell memory is approximately 8-15 years.[26,30] Unlike the antiviral $CD4^+$ T-cell response that is relatively uniform in terms of decay kinetics, antiviral $CD8^+$ T-cell responses appeared to split, with approximately half of the VV-immune population losing detectable $CD8^+$ T-cell memory at some time in the 20 years postvaccination. Further studies are needed, but it is possible that by using multiple cytokine combinations for estimating memory T-cell frequencies, we may find $CD8^+$ T-cell memory maintained in a larger proportion of subjects than the number determined based only the frequency of $IFN\gamma^+TNF\alpha^+$ T cells.[26]

measure the duration/half-life of human T-cell responses following smallpox vaccination. In one study, VV-specific T-cell responses measured mainly by IFNγ ELISPOT analysis showed that T-cell memory was detectable for >50 years while delcining slowly with a half-life of ~14 years.[30] Likewise, we measured the frequency of $IFN\gamma^+TNF\alpha^+$ VV-specific memory T cells by ICCS and found that memory could be maintained for up to 75 years and that virus-specific $CD4^+$ and $CD8^+$ T-cell responses declined with a half life of approximately 8-15 years[26] (Fig. 2). Despite using different T-cell quantitation techniques, these independent studies were in close agreement in terms of the estimated half-life of long-term T-cell memory. However, these studies were focused primarily on memory T-cell responses analyzed several years after vaccination and further studies have now examined the earlier kinetics of VV-specific T-cell responses.[31-35] Interestingly, primary antiviral T-cell responses in most human subjects peak between 14-21 days after VV infection,[31-35] which is a substantial delay compared to VV-specific T-cell responses in mice, which peak within the first 7 days after infection.[18] Another interesting observation revealed by these studies[31-35] is that antiviral $CD8^+$ T-cell responses decline dramatically over the course of the first few weeks/months after the infection has cleared before reaching a more stable, albeit slowly declining plateau phase of immunological memory. Comparing studies that examined the early VV-specific T-cell response[31-35] to the studies that focused on long-term T-cell memory,[26,30] it appears that antiviral T-cell responses may decline in a biphasic manner; a rapid initial decline in virus-specific T-cell

numbers followed by a slower decline in T-cell memory at later time points. With this in mind, it is possible that the 8-15 year half life of T-cell memory following VV infection[26,30] may be an overly conservative estimate and once these different decay rates are separated, the duration of late-stage T-cell memory at >1 year post-infection may be longer than currently estimated.

Analysis of virus-specific $CD4^+$ T-cell memory versus $CD8^+$ T-cell memory following VV infection has also revealed some interesting differences between these two T-cell subsets. Although the long term T-cell half life estimates are similar ($T_{1/2}$ = 8-12 years and $T_{1/2}$ = 8-15 years for $CD4^+$ and $CD8^+$ T cells, respectively), $CD4^+$ T-cell memory appeared to be maintained more efficiently than $CD8^+$ T-cell memory since nearly half of VV-immune subjects lost detectable $CD8^+$ T-cell responses within 20 years after vaccination whereas $CD4^+$ T-cell responses declined but remained readily detectable in most individuals during the same observation period.[26] Similar results were found by an independent group[33] and this observation may be due, at least in part, to strikingly different dynamics in the early kinetics of the T-cell response following VV infection. When compared side-by-side, VV-specific $CD4^+$ T-cell numbers do not reach the same peak levels as the coinciding $CD8^+$ T-cell response, but they also do not decline as sharply as their $CD8^+$ T-cell counterparts during the first weeks/months after infection.[33,35] This indicates that the kinetics and relative magnitude of human $CD8^+$ and $CD4^+$ T-cell subpopulations differ substantially following this acute viral infection and a better understanding of why these differences exist will be an important area of future investigation.

Lifelong immunity occurs following childhood infection with MV and this was perhaps most clearly demonstrated by Panum[36] who showed that following a MV epidemic in 1781, isolated inhabitants on the remote Faroe Islands were protected against reinfection during a second outbreak that occurred 65 years later in 1846. Since the island was not visited during the intervening years between these two outbreaks, it appears that antiviral immunity persisted in the absence of environmental re-exposure. It is believed that strong T-cell responses are important for protection against MV[37] and several techniques have been used to measure MV-specific T-cell responses including direct ex vivo CTL assays, ELISPOT, ICCS and peptide-MHC Class I tetramer staining.[37-40] One study[41] examined the relative levels and duration of MV-specific T-cell memory following 10 days of in vitro expansion of carboxyfluorescein succinimidyl ester (CFSE)-labeled lymphocytes. Following this period of expansion, MV-reactive $CD4^+$ and $CD8^+$ T-cell responses could be detected for up to 34 years after vaccination and interestingly, the study suggests that $CD8^+$ T-cell memory remained largely intact whereas $CD4^+$ T-cell responses appeared to decline over time. This appears to be different from the results observed following VV infection wherein $CD8^+$ T cells initially decline more rapidly than $CD4^+$ T cells and then at later time points both populations decline slowly with similar decay rates. Further studies will be needed to determine if $CD4^+$ and $CD8^+$ T-cell memory responses following VV infection represent a paradigm for T-cell responses to other acute viral infections or whether each viral infection instead triggers T-cell memory with different long-term kinetic patterns for $CD4^+$ and $CD8^+$ T-cell subsets.

Humoral immunity against YFV can be maintained for up to 75 years after infection[42] but there is relatively little known about the overall duration of YFV-specific T-cell responses. One longitudinal study identified stable T-cell memory by IFNγ ELISPOT analysis for up to 18 months after YFV infection[43] but another more recent study by Miller et al[35] has provided further insight into the kinetics and magnitude of the early phases of the antiviral T-cell response to YFV. In this study, antiviral T-cell responses against YFV and VV were compared using multiple quantitation techniques including peptide-MHC Class I tetramer staining, ICCS and two sets of phenotypic markers that identified activated T-cell populations. As indicated in Figure 3, peptide-MHC Class I tetramer staining identifies a small defined T-cell population with specificity to only a single peptide epitope bound to one MHC haplotype. Use of IFNγ ICCS (or ELISPOT) allows T cells of multiple antigenic specificities to be identified by using peptide pools or infected APC for stimulation. If more than one cytokine is used for T-cell quantitation, then an even larger frequency of virus-specific T cells can be identified because it will include T-cell populations that do not

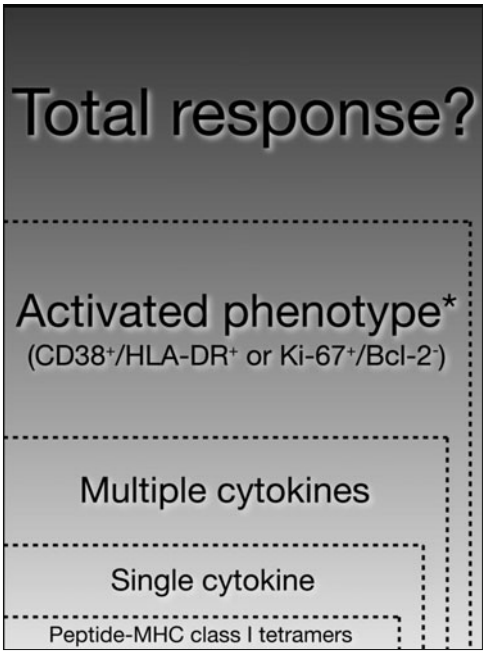

Figure 3. Comparison between different quantitation approaches used to measure T-cell memory. The measured frequency of antigen-specific T cells depends to a great extent on which techniques are used for their quantitation. This figure shows an illustration of the proportion of a virus-specific T-cell response that can be detected using currently available techniques. Peptide-MHC Class I tetramer analysis is highly specific, but identifies the lowest percentage of the total virus-specific T-cell response due to measuring only T cells with a single peptide specificity. Measuring a single cytokine such as IFNγ by ICCS allows identification of a potentially broader subset of T cells than using a peptide-MHC Class I tetramer due to the ability to use pools of peptides, entire viral antigens or virus-infected APC. Performing ICCS that is based on the production of multiple cytokines may further increase the number of virus-specific T cells that are measured due to identification T-cell populations that may not produce any one cytokine of interest. For instance, several studies have found virus-specific T-cell populations that fail to produce IFNγ, the most common cytokine used for measuring T-cell memory. T-cell quantitation based on phenotypic analysis using markers such as $CD38^+HLA\text{-}DR^+$ or $Ki\text{-}67^+Bcl\text{-}2^-$, is capable of detecting the highest proportion of activated T cells during the early stages of infection. Although we may never know the "total" T-cell response mounted during acute human infection, the various assays described here provide the initial steps towards achieving this goal.
*Note: Estimating an antiviral T-cell response based on phenotypic markers ($CD38^+HLA\text{-}DR^+$ or $Ki\text{-}67^+Bcl\text{-}2^-$) is only valid during the first few weeks after infection since these activation markers are rapidly down-regulated on virus-specific T cells at later time points.

produce IFNγ directly ex vivo. In addition to these antigen-specific assays (peptide-MHC Class I tetramer binding or cytokine production), analysis of virus-induced T-cell populations can also be indirectly estimated by measuring the frequency of T cells bearing an activated $CD38^+HLA\text{-}DR^+$ or $Ki\text{-}67^+Bcl\text{-}2^-$ phenotype.[35,44] Using either of these phenotypic marker combinations, it appears that there are ~3-fold more T cells elicited by VV and YFV infection than what are measured using other current approaches to T-cell quantitation. Based on the Miller study,[35] the frequency of virus-specific T cells at the peak of the antiviral immune response could reach as high as 12% to 40% of total peripheral T cells following YFV or VV infection, respectively. This is intriguing

because it indicates that human T-cell responses to acute viral infection may be far more robust than previously realized.[35,45] These studies also showed that YFV-specific T-cell kinetics mimic the responses observed following VV infection, with antiviral T-cell responses peaking by ~14 days after infection and then dropping rapidly before reaching the memory phase of the immune response within 1-6 months after infection.

Functional Attributes of Human Memory T Cells

Concerns with bioterrorism and the threat of natural emerging/re-emerging infectious disease has greatly accelerated our understanding of human T-cell immunology. As noted above, we have gained considerable information in terms of the induction and maintenance of immunological memory. We have also made substantial progress in understanding the basic antiviral functions of human $CD4^+$ and $CD8^+$ T cells and their role in antiviral immunity. The NIH funded a program entitled, "Large Scale B- and T-cell Epitope Discovery" and this has led to an explosion of research dedicated to identifying $CD4^+$ and $CD8^+$ T-cell epitopes in a variety of human pathogens (see http://www.immuneepitope.org). For orthopoxvirus research in particular, this has revolutionized the field. Over 170 human and murine T-cell epitopes have been mapped in VV[46] and this has led to the opportunity to ask immunological questions that would have otherwise been unfeasible. Is T-cell immunodominance a factor in shaping the immune response to a complex virus in outbred human populations? What are the attributes of highly immunogenic viral proteins? Are early gene products targeted more often than late genes? With a toolbox of mapped $CD4^+$ and $CD8^+$ T-cell epitopes in hand, we are beginning to find answers to these fundamental questions.

T-cell immunodominance occurs when the majority of the T-cell response is directed to only a small number of potential peptide epitopes. VV represents a large DNA virus with approximately 180 predicted open reading frames (ORFs) and the potential to harbor many peptide epitopes. Close to 120 human $CD8^+$ T-cell epitopes have been mapped across 103 VV ORFs, making it clear that the antiviral $CD8^+$ T-cell response following this acute viral infection is remarkably broad.[46] This is not just a characteristic of VV since $CD8^+$ T-cell responses to MV are also diverse and one study mapping HLA-A2-restricted T cells found that no single peptide dominated the T-cell response.[40] Similar to VV-specific $CD8^+$ T-cell responses, VV-specific $CD4^+$ T-cell responses are surprisingly diverse with $CD4^+$ T cells recognizing 122 different VV ORFs.[47] On average, each subject developed $CD4^+$ T-cell responses against 39 VV ORFs with a range of 13 to 63 VV ORFs. In comparison, a proteome-wide analysis of antiviral $CD4^+$ T-cell responses to human cytomegalovirus (CMV) revealed a median of 12 CMV ORFs with as many as 39 CMV ORFs being recognized by $CD4^+$ T cells.[48] Although these viruses are similar in genome size and number of ORFs, they differ significantly in the sense that VV induces only an acute infection that is rapidly cleared whereas CMV induces a chronic and lifelong infection.

A meta-analysis of viral protein immunogenicity was performed based on 8 studies that together included 151 human and murine $CD8^+$ T-cell epitopes spanning 62 VV ORFs.[46] Based on VV protein expression kinetics, 47% of the $CD8^+$ T-cell response targeted early genes, 19% targeted late genes and the remaining 34% of the response targeted genes with unknown kinetics or both early and late kinetics. Although not absolute, these results indicate that $CD8^+$ T cells show a trend toward preferentially targeting early gene products. Comparisons between viral proteins divided according to functional attributes (e.g., replication/viral regulation, virulence/host range or structural) did not reveal a clear preference in $CD8^+$ T-cell recognition in this meta-analysis. Comparison of protein localization indicated that nearly half (48%) of the $CD8^+$ T-cell response targeted intracellular proteins with 12% of the T cells targeting membrane proteins, 8% targeting secreted proteins and 32% of the response directed towards proteins of unknown localization. In a large proteomic analysis of $CD4^+$ T-cell responses to VV, the most commonly targeted ORFs included structural proteins and proteins with late expression kinetics.[47] There was also a trend towards higher recognition of larger vs smaller virus proteins.[47] Similar results were observed after mapping murine T-cell epitopes in VV-infected

mice showing that $CD8^+$ T-cell responses tended to target early gene products whereas $CD4^+$ T-cell epitopes showed a modest trend towards recognition of late gene products (39% early, 61% late gene recognition by $CD4^+$ T cells, respectively).[49] Together, this indicates that T-cell responses to a complex virus such as VV is broad and targets a wide variety of proteins based on localization, structure/size and time of gene expression, which together is likely to provide efficient recognition of infected cells during the course of acute viral infection.

Perhaps the most important aspect of T-cell memory is the ability to express a variety of antiviral effector molecules upon cognate interactions with their specific peptide antigen. VV-specific T cells produce a number of different cytokines including IFNγ, TNFα, IL-2, IL-4, IL-13 and MIP1β,[33,50,51] costimulatory adhesion molecules such as CD40L[51,52] and cytolytic molecules such as granzyme A, granzyme B and perforin.[35,50,53,54] The expression pattern of different effector molecules depends on the T-cell subpopulation (CD4 vs CD8) as well as the time point examined after infection, since expression profiles often change dramatically between the peak of the antiviral T-cell response and the resting memory stages of the immune response. Although both $CD4^+$ and $CD8^+$ T cells have the ability to express IFNγ, TNFα and IL-2, only VV-specific $CD4^+$ T cells express the Th2 cytokine, IL-13.[33] IFNγ is the most common cytokine used to measure T-cell responses and in murine models there is essentially a 1:1 ratio between $IFN\gamma^+$ $CD8^+$ T cells and peptide-MHC Class I $tetramer^+$ T cells.[16] With human T cells (especially human $CD4^+$ T cells), it is becoming clear that IFNγ production may identify only a subpopulation of the total virus-specific T-cell response. Some studies have identified VV-specific $CD4^+$ T-cell clones that proliferate against VV antigens, but fail to produce IFNγ.[55] Likewise, other studies have identified primary VV-specific $CD4^+$ T cells that were IFNγ-negative but still produced other cytokines including TNFα, IL-2, or IL-13 in response to VV stimulation.[33,44] Indeed, analysis of IFNγ, TNFα and IL-2 production by VV-specific $CD4^+$ T cells revealed T-cell subpopulations producing each of the 7 possible combinations of these 3 cytokines.[51] Compared to $CD4^+$ T cells, VV-specific $CD8^+$ T cells are more likely to express IFNγ. However, detailed analysis of cytokine profiles including IFNγ, TNFα, IL-2 and MIP1β also demonstrate the existence of VV-specific $CD8^+$ T-cell subpopulations that express a variety of cytokines in the absence of IFNγ production.[50] These variations in cytokine expression patterns may explain the dichotomy observed in long-term $CD8^+$ T-cell responses measured by Hammarlund et al.[26] In those studies, polyfunctional $CD4^+$ and $CD8^+$ T-cell responses were measured on the basis of dual production of two cytokines, IFNγ and TNFα. Although $CD4^+$ T-cell responses appeared fairly uniform in their decay rates, $CD8^+$ T-cell responses split into two groups by 20 years postvaccination wherein about half of VV-immune individuals maintained detectable $CD8^+$ T-cell memory and the other half of the sample population appeared to lose detectable VV-specific $CD8^+$ T-cell responses (Fig. 2). Retesting of a subset of these samples has revealed that many of the VV-specific $CD8^+$ T cells examined at >20 years after infection had not actually disappeared, but instead appear to make other cytokine combinations besides IFNγ and TNFα (Slifka and Hammarlund, unpublished results) and were not previously identified because they did not produce both IFNγ and TNFα. This emphasizes the point illustrated in Figure 3 indicating that it is important to measure as many cytokine combinations as possible when quantitating T-cell memory because measuring any cytokine alone is likely to lead to a conservatively lower estimate of the total antigen-specific T-cell response.

Perforin and granzyme B are expressed by nearly all VV-specific MHC Class I tetramer-positive $CD8^+$ T cells at early time points after infection.[35] Interestingly, <8% of VV-specific $CD8^+$ T cells express perforin directly ex vivo by one month after infection.[53] However, the memory T cells are able to re-express perforin following 7 days of in vitro restimulation, indicating that although this important cytolytic molecule is rapidly downregulated in vivo after VV infection has cleared, it can be quickly upregulated after re-exposure to specific viral antigens.[35] In contrast to perforin, other cytolytic molecules such as granzyme A and granzyme B continue to be expressed in a sizeable subpopulation of MHC Class I tetramer-positive or restimulated $IFN\gamma^+$ $CD8^+$ T cells at 1 month post-infection.[35,50,53] The proportion of $CD8^+$ T cells expressing

both granzyme A and granzyme B declines from 60% at one month to 33% of the virus-specific T-cell response at one year post-infection.[53] This is still a relatively high percentage of memory cells expressing granzymes when compared to perforin expression, which has dropped to nearly baseline levels within the first month after infection. This indicates that, similar to shifting virus-specific cytokine expression profiles, cytolytic proteins such as perforin and granzymes A and B are differentially regulated after acute viral infection.

In contrast to $CD8^+$ T cells, there is much less known about the kinetics and expression levels of cytolytic proteins in cytotoxic $CD4^+$ T cells. Although most cytotoxic T-lymphocytes are $CD8^+$ T cells, it is important to note that cytotoxic $CD4^+$ T cells have been identified directly ex vivo following human MV infection[38] and the development of cytolytic $CD4^+$ T-cell responses against acute and chronic viral infections are far more common than one might expect. Virus-specific $CD4^+$ CTL are MHC Class II-restricted[56-62] and have been identified following infection with MV,[38] VV,[59-62] polio,[63] dengue,[64] influenza,[57] hepatitis B virus,[58] varicella zoster virus,[65] Epstein Barr virus,[57,66] herpes simplex virus[56] and CMV.[67,68] Although MHC Class II (e.g., HLA-DR) is typically expressed on professional APC, following infection and the resulting inflammatory cytokine response, MHC Class II is upregulated on nonprofessional APC including human epithelial cells[69-74] as well as highly activated virus-specific T cells[35] and potentially other cell types as well. This indicates that during acute infection, cytolytic $CD4^+$ T cells may be capable of enhanced immune surveillance due to the transient upregulation of MHC Class II on a broader array of host cells.

Conclusion

Over the last several decades a number of different techniques have been developed to assess the duration and functional characteristics of T-cell-mediated immunity following acute viral infection. These techniques have been refined to permit the detection of precisely defined, low frequency antigen-specific T-cell subsets directly ex vivo. Analysis of T-cell memory following infection with VV, MV or YFV has provided valuable insight into the kinetics, magnitude and duration of virus-specific T-cell responses. $CD4^+$ and $CD8^+$ T-cell memory has been demonstrated up to 75 years after VV infection and 34 years following MV infection. Although the half-life of VV-specific T cells has been calculated at 8-15 years following VV infection, it is unclear if this degree of immunological memory is representative of other acute viral infections or if this is specific only to VV infection. Likewise, more information on the relative duration of $CD4^+$ versus $CD8^+$ T-cell memory following acute viral infection is needed in order to determine if there are virus-specific patterns of T-cell memory or if the immune response to a variety of viruses is similar. The expression of effector cytokines and cytotoxic proteins has been shown to evolve throughout the course of primary viral infection and memory T-cell generation. This effect can be observed in shifting cytokine production profiles of virus-specific T cells as well as the differential regulation of perforin compared to granzyme A and granzyme B. The biological relevance of these various patterns of effector molecule expression in the context of acute human infection have yet to be fully understood.

Analysis of T-cell memory following acute infection by viruses such as VV, MV and YFV illustrate both the great strides that have been made in our knowledge of T-cell-mediated immunity as well as the sizeable gaps that remain in our understanding of human T-cell immunobiology. It will be exciting to learn the mechanisms that govern the longevity of memory T cells induced by acute viral infection and learn how to best mimic these forms of immunological memory by developing improved vaccines that elicit effective and long-lived T-cell responses.

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Chapter 9

Principles of Memory CD8 T-Cells Generation in Relation to Protective Immunity

Maurizio Zanetti,* Paola Castiglioni and Elizabeth Ingulli

Abstract

Memory T-cell responses are of vital importance in understanding the host's immune response against pathogens and cancer cells and to begin establishing the correlation of protection against disease. In this review, we discuss our own data in the general context of current knowledge to sketch tentative working principles for the induction of protective T-cell responses by vaccination. We draw attention to quantitative and qualitative aspects of the initial contact with antigen, as well as to the kinetics of events leading to the generation of memory T cells thereafter. Our arguments are based on the current distinction of memory T cells into two lineages: effector memory T cells (T_{EM}) and central memory T cells (T_{CM}). Our provisional conclusion is that protective T-cell responses correlate positively with the T cells of the central memory phenotype. In proposing a set of working principles to enable protective memory T cells by vaccination we address vaccination both in the context of the immunologically-inexperienced and immunologically-experienced individual, respectively. Finally, we draw attention to the interplay between systemic and local immunity as important factors in determining the success of memory T-cell responses in protecting the individual. We believe that considerations on the immunodynamics of memory induction and maintenance, memory lineage differentiation and their relation to protection may help design strategies to control disease caused by pathogens and cancer.

Introduction

Remembrance of things past is a general mechanism of experience organization and function programming.[1] Immunological memory spans across the lifetime of the individual and the inability to establish immunological memory could be seen as a deleterious event for the evolution of the species. A general mechanism of experience organization and function programming for immunological memory involves the acquisition and maintenance of previous information in relation to the same antigen. The study of immunological memory implies revealing the link(s) between single elements of the immune system (i.e., lymphocytes) and functional topology to explain how longevity and protective function are generated. In molecular terms, this equates to a better understanding of the enablement of gene programs and transcriptional events in T cells.

Previously, we defined immunological memory as the event that occurs when the immunologically experienced individual reencounters antigen through infection, tumor growth, or vaccination and develops an even greater response than after the first exposure to the same antigen.[2] Studies

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show that immunological memory owes its characteristics to an increased frequency of specific B and T-lymphocytes. Furthermore, it appears that T cells have heightened sensitivity for antigen[3] and B cells have high susceptibility to activation and proliferation in an antigen-independent way by microbial products.[4]

At the present time, vaccines for use in humans owe their effectiveness to the induction of neutralizing and opsonizing antibodies[5,6] working to intercept pathogens at the port of entry, in the blood stream or in the intercellular space depending on the pathogen's pathway for infection. For instance, pathogens that initially come in contact with external secretions (e.g., influenza virus), or enter the bloodstream in a cell-free form (e.g., polio virus) are intercepted by antibodies that prevent infection or disease, respectively. Protection by memory antibody responses is also subject to the length of the incubation period. A pathogen with a short incubation period (< 3 days, as for the influenza virus) requires that protective levels of serum neutralizing antibody be present at the time of exposure to prevent the establishment of infection.[7] The portal of entry, in this instance, is also the site of pathology in the respiratory tract, and the degree of resistance to influenza virus infection is directly proportional to the level of specific hemagglutination-inhibition antibody in the secretion of the respiratory tract.[8] Therefore, to prevent influenza infection it is necessary to induce and maintain antibody titers above levels associated with protection against appropriate type-specific variants. To this end, vaccines need to be reformulated periodically to compensate for antigenic shift and drift. However, as it will be discussed below, an alternative to this is to elicit cytotoxic T-cell responses against conserved determinants of the virus such as the matrix and nucleoprotein antigens, so to obviate the constant need to chase antigenic variations of the virus. For diseases of longer incubation period, e.g., paralytic poliomyelitis (>3 days for central nervous system invasion from the primary site of infection), immunologic memory enables durable resistance to paralysis.[7] Recently, new clues on the generation of memory antibody responses have begun to emerge. Working in the influenza system Wilson et al[9] demonstrated that approximately 7 days after the booster vaccination there is a rapid and robust influenza specific IgG1 antibody-secreting plasma cell response accounting for up to 6% of peripheral blood B-cells. This is consistent with the fact that memory antibody responses could result either from long-lived plasma cells in the bone marrow[10] or from a de novo differentiation of antigen-specific B-cells into plasma cells following polyclonal activation.[11]

For many diseases (e.g., measles, mumps, rubella and smallpox), immunity conferred by infection or vaccination provides life long immunity. Based on anecdotic recounts and scientific observations, the longevity of memory responses against some major infectious agents has been estimated to be between 40 and 75 years.[12-15] Thus, immunity by natural infection or vaccination with inactivated or attenuated virus establishes life long immunity. Since an effective anamnestic response to many pathogens does not need periodic reinforcement by immunization, it appears as if in the course of evolution, the primary antibody response evolved to clear the host of the invading organisms, while the memory antibody response evolved to prevent re-infection.

Compared with memory antibody responses much less is known about the parameters for induction and maintenance of memory T cells and particularly their role in protection. Our goal in this chapter is to discuss our views on this issue and point to a few general principles on the link between memory T cells and protection by vaccination. Ultimately, this may benefit the design of the next generation of vaccines against viruses, parasites and cancer cells.

T-Cell Immunity—From Activation to Imprinting T-Cell Memory

The defense against intracellular pathogens and cancer cells requires T-cell immunity. Seen in evolutionary terms, it appears as if memory T-cell responses evolved as a specialized mechanism for protection against intracellular pathogens that would have otherwise harmed the species irreparably. The effect of tumor cells is much more relevant today as the life span of humans has lengthened considerably due to better sanitation, antibiotics, and antiviral therapies. Like B-cell responses, primary T-cell responses reflect clonal selection and expansion[16] and are regulated by antigen.[17] Both naïve and memory T cells only recognize antigen processed and presented by specialized cells,

dendritic cells, macrophages, and B-lymphocytes.[18] In addition to peptide/MHC complexes at the surface of antigen presenting cells (APCs) activation of naïve T cells requires a positive signal from a second set of molecules, costimulatory molecules. Lack of costimulation prevents activation and promotes anergy.[19] Pathogens have evolved to display a rich set of molecules that can trigger the upregulation of costimulatory molecules on the APCs.[20-22] Unlike microbial pathogens, cancer cells lack costimulatory molecules and vaccines require the help of immunological adjuvants to exploit their immunogenic potential.

The primary T-cell response to infection or vaccination has temporal characteristics that are well understood (Fig. 1A). For instance, antigen-specific CD8 T cells go through clonal

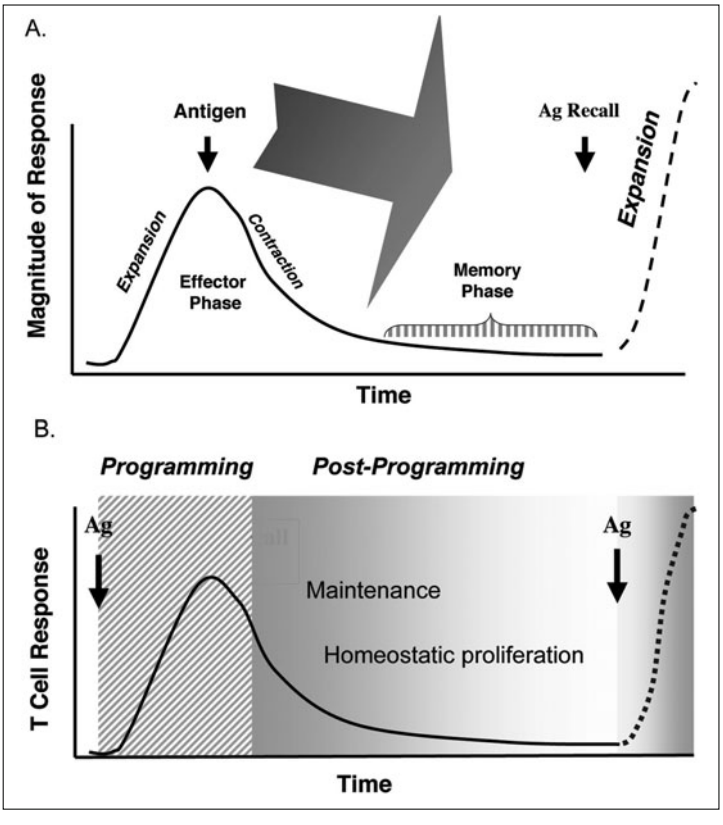

Figure 1. A) Schematic representation of the course of a primary T-cell response and relation between the events taking place during priming and the establishment of long-term memory T-cell responses. In response to primary infection or vaccination, antigen-specific T cells undergo a first phase of clonal selection and expansion followed by a contraction phase in which the great majority (~90%) of activated effector T cells undergo apoptosis. During this event the immune response slowly progresses into the emergence of memory T cells and their maintenance through homeostatic proliferation. This is the origin of a reservoir of antigen specific T cells expandable upon re-encounter of antigen. It has been estimated that 100 naïve CD8 T cells expand to 3×10^6 cells at the peak of the response around day 7 and then contract to 10^5 14 days after the initial contact with antigen. B) Schematic representation of the two main phases of the primary expansion leading into a long-term response awaiting for the reencounter with antigen. The programming and postprogramming phases and their relationship with maintenance and homeostatic proliferation are shown.

expansion and then contract so that the majority (~90%) of activated effector T cells die by apoptosis.[23,24] This happens with great regularity within the first 7-10 days from the initial contact with antigen.[25,26] At the end of this event, the immune response slowly progresses into memory CD8 T cells. These self-renew through a slow process termed homeostatic proliferation[27] and form a reservoir of resting memory CD8 T cells expandable upon re-encounter of antigen. Interestingly, both clonal expansion and contraction seem to be developmentally programmed and while the magnitude of clonal expansion is proportional to the dose of antigen and the strength of antigen presentation (APCs abundance, inflammation, degree of costimulation, etc),[28,29] contraction proceeds independent of the magnitude of the expansion.[30] A prevailing school of thought is that after priming, antigen is no longer required to maintain the pool of expandable memory T cells.

Given these general premises on the structural organization of the early T-cell response we shall begin by asking the question: "What are the requirements for the induction and maintenance of purposeful immunity?" The argument we intend to develop herein, central to our views on T-cell memory, is that the characteristics of the primary T-cell response dictate the type of immunity that will ensue after the initial burst, both in quantitative and qualitative terms, i.e., the longevity biological characteristics of T-cell memory. Our proposal is that the way priming occurs has a direct impact on the function of memory T cells and their ability to control disease (Fig 1B). Ultimately, understanding the rules of engagement will point us to effective T-cell vaccines.

Considerations on the Parameters of the Primary Response That Influence the Generation of Memory T Cells

These issues have been dealt in detail recently.[1,31] The basis for the proposed principles are recapitulated in Box 1.

Early observations showed that small doses of antigen favor cell-mediated responses and large doses of antigen favor antibody-mediated responses.[32,33] Too much antigen also activates a large fraction of available T-cell precursors and this could cause their deletion by exhaustion. Since the magnitude of the expansion of effector T cells directly correlates with the amount of

Box 1. Proposed parameters of the primary response that influence the generation of memory T cells

- Among adaptive memory CD8 T-cell responses, CD8 T_{CM} cells offer the best correlate of protection.
- Protective T-cell responses can be programmed if priming is induced in such a way as to respect the parameters that control the induction of T_{CM} cells.
- Expansion of memory T cells is an acquired property that correlates with the availability of T-cell help at the time of priming.
- Imprinting lineage selection, i.e., the generation and/or the selective expansion of T_{CM} vs T_{EM} cells, occurs at the time of priming by favoring the induction of memory T cells best fit for homeostatic proliferation and poised to long term persistence while retaining the ability to expand upon re-encounter with antigen.
- Successful imprinting must favor the induction and long-term maintenance of memory T cells under conditions that minimize the process of cell senescence after clonal expansion.
- Expansion of memory T cells upon antigen recall is an intrinsic property of memory T cells ($T_{CM} > T_{EM}$) not a reflection of their number prior to re-encounter with antigen.

antigen administered[28] we argue that excess antigen may not be necessary and even counteractive if the goal is to induce effective memory T cells. In line with this proposal are early studies on the relation between the priming dose and the induction of protective memory T-cell responses which established that protective CD4 T-cell (Th1) responses against the parasite *Leishmania major* in Balb/c mice were induced by priming with a low not high antigen dose.[3]

Although the magnitude of a primary CD8 T-cell response correlates with the infectious dose or the amount of antigen,[28] analysis of antigen dose at priming and the magnitude of the expansion phase of the memory response shows instead an inverse correlation.[30,35]

Another paradox to which reference has been made is whether antigen is required for the maintenance of memory T cells. Once set in motion by sufficient antigen a T-cell response has been shown to function on "autopilot".[36] We have argued that this may not necessarily be the case since protective T-cell memory responses against pathogens such as *Plasmodium malaria*,[37] *Leishmania major*,[38] Bacillus Calmette-Guerin[39] and LCMV[40] fade rather quickly when infection is eliminated by sterilizing chemotherapy. At variance with this view is that the long-term survival of memory T cells in vivo is apparently dependent on a nonspecific interaction with MHC molecules.[41]

From the foregoing it would appear that small quantities of antigen may be required to generate memory responses, arguing that memory T cells accounting for protection could emerge from T-cell exposed to scarce amounts of antigen and/or a discrete number of APCs.

A number of studies have shown that T-cell help plays an important role in determining the initial activation (programming phase) as well as destiny (post-programming phase) of CD8 T cells, that is the emergence and maintenance of memory T cells.[42-46] Helpless priming negatively affects the long-term number of resting memory CTL precursors during the maintenance phase of the memory response by a factor of 10[47] and program cells to undergo TRAIL-mediated apoptosis upon re-encounter with antigen.[48] Importantly, helpless priming creates unfavorable conditions for the generation of protective responses.[44,47]

It is also commonly accepted that the initial T-cell activation by the APC requires some degree of inflammation. This condition is thought necessary to up-regulate costimulatory molecules on the APC. However, contrary to this generally held belief we originally argued that overt inflammation may play adversely on the induction of T-cell memory.[1,31] Experiments show that inflammation during priming by way of concomitant infection with Listeria monocytogenes or by administration of Toll-like receptor agonists dramatically diminish the generation of antigen-specific memory CD8 T cells and severely restrict their expansion upon recall infection.[26] Similarly, tinkering with the CD40 receptor was found to abolish memory responses.[49,50] Thus, the common practice of taking advantage of an inflammatory umbrella to amplify effector T-cell expansion may curtail the generation of memory T cells. A difficulty we are confronted with is being able to distinguish, in quantitative and qualitative terms, the threshold of inflammation necessary to jump-start T-cell activation from that which seems to hinder the generation and maintenance of memory T cells. Studies will need to address this issue.

Not unexpectedly, high antigen dose, excess costimulatory activation, TLR triggering, and overt inflammation have a direct impact and take their toll on the replicative life of memory T cells pushing them into proliferative arrest, senescence.[51] Senescent T cells, like other somatic cells, have short telomeres and are end-stage differentiation memory T cells[52] that offer little if any value to ongoing or future immune responses. The progressive loss of immunological memory observed in ageing correlates with reduced frequency of antigen-specific IFN-γ producing cells[53] and proliferative capacity.[54,55] In chronic HIV infection excessive antigen stimulation drives cells in a state of replicative senescence with a prevalence of $CD57^+$ CD8 T cells.[54,56]

Memory CD8 T-Cell Subpopulations, Lineage Commitment and Protective Responses

To narrow our focus and to extend the analysis let us draw attention to the general idea that cells destined to become memory T cells are not only the survivors of activation-induced cell death but also undergo lineage differentiation to give origin to two sub-populations equivalent in the

mouse and in humans: effector memory (T_{EM}) and central memory (T_{CM}) cells. Their distinction is based primarily on the expression of L-selectin (CD62L) and the CC-chemokine receptor 7 (CCR7). These determine the capacity to home lymph nodes.[57,58] Their expression is elevated in central memory T cells and is minimal or absent in effector memory T cells. The characteristics that distinguish T_{EM} and T_{CM} cells include homing to lymph nodes, homeostatic and antigen-driven proliferative potential,[59] the ability to kill in vivo[60], and IL-2 production after antigen stimulation.[59] Together, this distinction may also be important in understanding how the two lineages of memory T cells originate and persist in the immunologically experienced individual and how they relate to protection.

The distinction between T_{EM} and T_{CM} cells is relevant not only to understand how and when memory T cells with characteristics of T_{EM} and T_{CM} cells are generated (the lineage differentiation problem) but also how they differ with respect to mediating protection in vivo. As for the lineage differentiation problem it was originally suggested that T_{CM} cells provide a continuous source of T_{EM} cells[61] even though TCRβ repertoire analysis shows that most T_{EM} cells may not derive from T_{CM} cells.[62] An alternate view posits that T_{EM} cells convert into T_{CM} cells according to a linear differentiation program where effector T cells are converted into T_{EM} cells and these into T_{CM} cells.[25,28] Accordingly, following the clonal expansion of effector T cells, T_{EM} and T_{CM} cells slowly emerge and differentiate.[59] A third view suggests that commitment to a particular memory T-cell lineage is governed by the initial naïve precursor frequency[63] so that at low precursor frequency, T_{CM} cells develop as a stable memory population preferentially. Interestingly, if the ratio APCs:precursor CD8 T-cell frequency is high, T_{CM} cells are virtually not generated.[63] The issue is no doubt complex since by comparing responses with different naïve precursor frequencies it was shown that the naïve precursor frequency influences the kinetics (not the magnitude) of the primary response and inversely correlates with the generation of CD62L^{hi} memory T cells.[64]

The inference to be drawn is that the two memory T-cell subpopulations are established according to a master program whereby a T_{EM} -> T_{CM} conversion would be the exception, not the rule, at physiological low precursor frequencies, and T_{CM} cells have the ability to develop as a stable lineage over a range of precursor frequencies and under conditions of low antigen presentation and seemingly low antigen dose.

Distinguishing memory T cells into two populations, T_{EM} and T_{CM} cells, have been key to shedding light on the correlation of protection. The first hint came from studies using as a model LCMV infection in which protection is known to correlate with memory not with effector CD8 T cells.[40] The first studies showed that viral replication in vivo was more effectively controlled by CD8 T_{CM} cells.[59] Work from this laboratory expanded the initial observation and is reviewed below.

Memory CD8 T Cells Induced by Low Antigen Dose Vaccination Protect Mice from Influenza a Virus Infection—The Role of CD62^{hi} Memory CD8 T (T_{CM}) Cells

In mice, as well as in humans, influenza virus infection is an inflammatory disease of the airway.[65] CTL responses are coordinately associated with attenuation of symptoms and protection from disease.[66,67] During natural infection the T-cell response peaks around day 7-10[68] and then contracts, while recovery from infection occurs, and T-cell memory is gradually generated and thereafter maintained.

As a model system of vaccination we used transgenic B-lymphocytes, a new way to program T-cell responses in vivo based on the fact that mature naïve B-lymphocytes can be effectively turned into powerful APCs with a dual capacity of synthesis and presentation of antigen to T cells in vivo following internalization of suitably engineered plasmid DNA.[69] A single intravenous injection of transgenic lymphocytes activates T-cell responses reproducibly and specifically even at very low cell doses ($<10^3$).

Mice were vaccinated by single intravenous injection of APCs (syngeneic B-lymphocytes) transgenic with plasmid DNA coding for the influenza virus peptide ASNENMETM ($NP_{366-374}$),

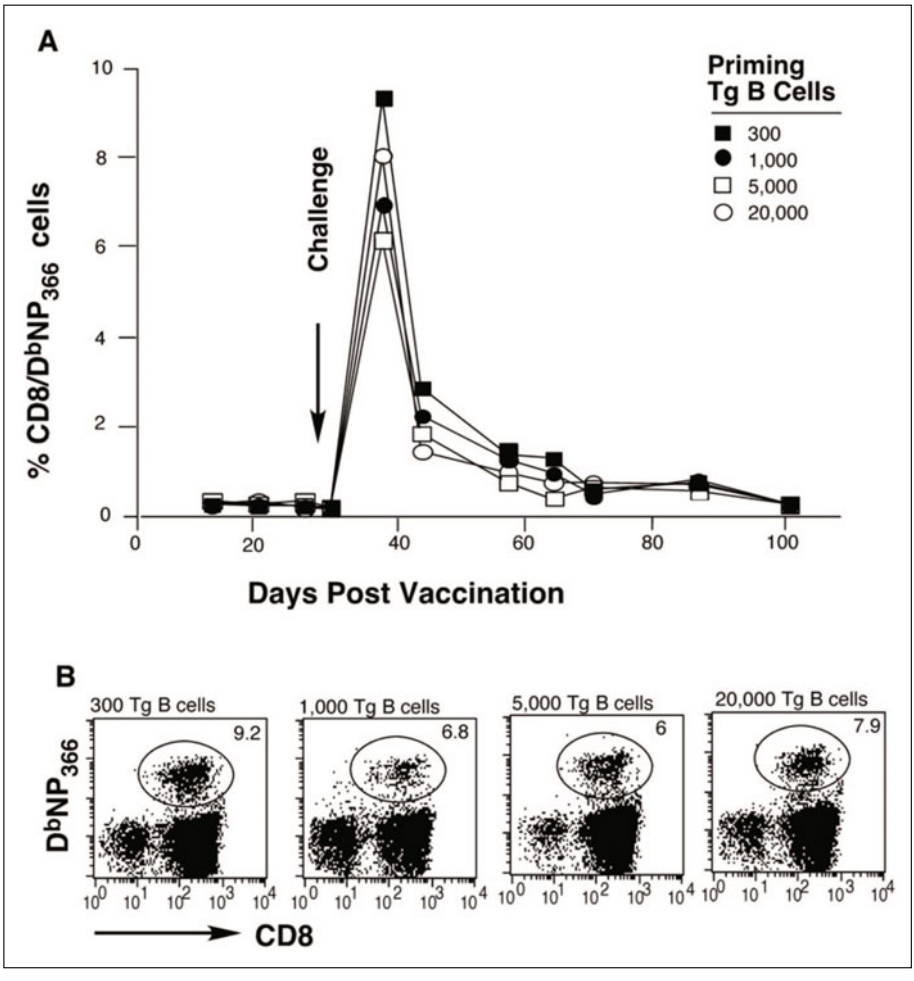

Figure 2. Analysis of the response in mice primed with different doses of transgenic B-lymphocytes shows that a small immunizing dose yields an expansion of memory CD8 T cells comparable to a high dose. A) Mice were primed by single injection of transgenic B-lymphocytes ranging from 3×10^2 to 2×10^4 cells/inoculum. Mice were challenged with a sub-lethal dose of A/PR8/34 influenza virus *i.n.* on day 28 after priming and peripheral blood lymphocytes were analyzed longitudinally as shown in the figure. B) Specificity of the staining with the $D^b/NP_{366\text{-}374}$ tetramer. Peripheral blood lymphocytes were collected on day 11 after challenge from groups of mice primed with different doses of transgenic lymphocytes (as indicated in each panel). Peripheral blood was pooled from each group (four mice per group) and cells were then stained with an anti-CD8 monoclonal antibody and $D^b/NP_{366\text{-}374}$ tetramer. The percentage of $D^b/NP_{366\text{-}374}$ tetramer specific CD8 T cells is indicated in each panel. (Reproduced from: Castiglioni P et al. Vaccine 2004; 23:699-708;[72] with permission.)

an epitope restricted by D,[b70] under a B-cell specific promoter. Vaccination induced systemic anti-virus CD8 T cells immunity[71] even though tetramer positive CD8 T cells could not be detected during the primary response, suggesting that the genetically engineered APCs provide a relatively weak immunogenic stimulus. This could be due to either low abundance of antigen, low number of APCs, or little pro-inflammation. Nonetheless, immunization by single intravenous

injection of APCs induced a durable CTL response that protected mice from an intranasal lethal virus challenge 21 or 60 days after vaccination.[71] The protective effect of vaccination could be generated with a small ($<10^3$) APC priming dose.[72]

To gain insight into the events of protection we decided to monitor the expansion of memory CD8 T cells after a sub-lethal virus challenge. The expansion of $D^b/NP_{366-374}$ tetramer-positive CD8 T-lymphocytes occurred at all doses tested with a magnitude comparable to that following priming with virus (Fig. 2). In other terms, there appeared to be no advantage in priming with a high antigen dose (a large APC inoculum) if the purpose was to induce memory CD8 T-cell responses that could be easily expanded upon re-encounter with antigen. We, then, decided to use this system to assess which sub-population of CD8 memory T cells were responsible for in disease protection. To this end, CD8 T cells were purified on the basis of $CD62L^{hi}$ and $CD62L^{low}$ from transgenic B-lymphocyte-primed or virus-primed mice 21 days after priming and adoptively transferred (10^6 CD8 T cells/inoculum) into naïve C57Bl/6 recipients (Fig. 4A). Mice were given a lethal virus challenge 48 hours later. Thirteen out of 15 (87%) mice adoptively-transferred with $CD62L^{hi}$ cells survived, whereas transfer of $CD62L^{low}$ cells protected only 3 out of 9 (33%) mice ($p = 0.019$). Similarly, 5 out of 8 (63%) mice given $CD62L^{hi}$ cells from live virus-primed mice were protected, whereas the transfer of $CD62L^{low}$ cells was ineffective (0 out of 4). Thus, protection from disease in the case of acute influenza virus infection is property of $CD62L^{hi}$ CD8 memory T cells.[72]

Central Memory CD8 T Cells Correlate with Protection against SIV in Rhesus Macaques

In collaboration with Dr. Genoveffa Franchini (NIH/NCI) we had the opportunity to verify the validity of the new working principle in Rhesus macaques vaccinated and exposed to the simian immunodeficiency virus (SIV). These studies are reviewed in detail in the chapter by Dr. Franchini in this book. The hypothesis that CD8 central memory T cells correlates with protection (for that purpose viremia was chosen as the end point) was tested on archival blood samples so that the results were not biased by vaccine use and vaccination design. The study consisted in measuring longitudinally the relative frequency of CD8 T_{CM} and T_{EM} cells specific for Gag and Env. It was found that the level of SIVmac251 replication following challenge exposure correlated inversely with the magnitude of vaccine-induced virus specific $CD8^+$ central memory T cells but not with $CD8^+$ effector memory T cells suggesting that CD8 T_{CM} cells play a pivotal role in the control of viral replication.[73] This study also made it clear that the quality of vaccine-induced $CD8^+$ T cells matters more than the magnitude of the total T-cell response itself. Paradoxically, in nonhuman primates a DNA vaccine is more effective than a vaccinia virus vaccine in generating protective memory response against SIV[74,75] even though the expression of antigens differs substantially in these two vaccine platforms.

Principles for Programming Protective T-Cell Responses by Vaccination in the Immunologically Inexperienced Individual

The experimental data that have been proposed thus far in the discussion point to the general idea that by controlling priming in qualitative and quantitative terms, one imprints the characteristics of the memory T-cell response. It is as if the power to control priming is the power to define the type of memory T-cell response required for protection against disease. The working principles for vaccination of the immunologically inexperienced individual are summarized in Box 2. Optimal induction of protective memory T-cell responses should be induced with low antigen dose and prolonged antigen presentation as these procedures (i) favor the induction of memory T cells that most readily expand to recall antigen, (ii) control the APCs:T-cell precursors ratio, (iii) protect against exhaustion and reduce the toll of senescence on the responding T cells, and (iv) direct lineage commitment in favor of T_{CM} cells. Priming should be under the umbrella of T-cell help to favor long-term survival of memory T cells and their expansion upon antigen recall, including protective responses. Although a direct role for T-cell help in the T_{CM}—T_{EM} lineage differentiation has not been directly analyzed, the available data imply that T-cell help is

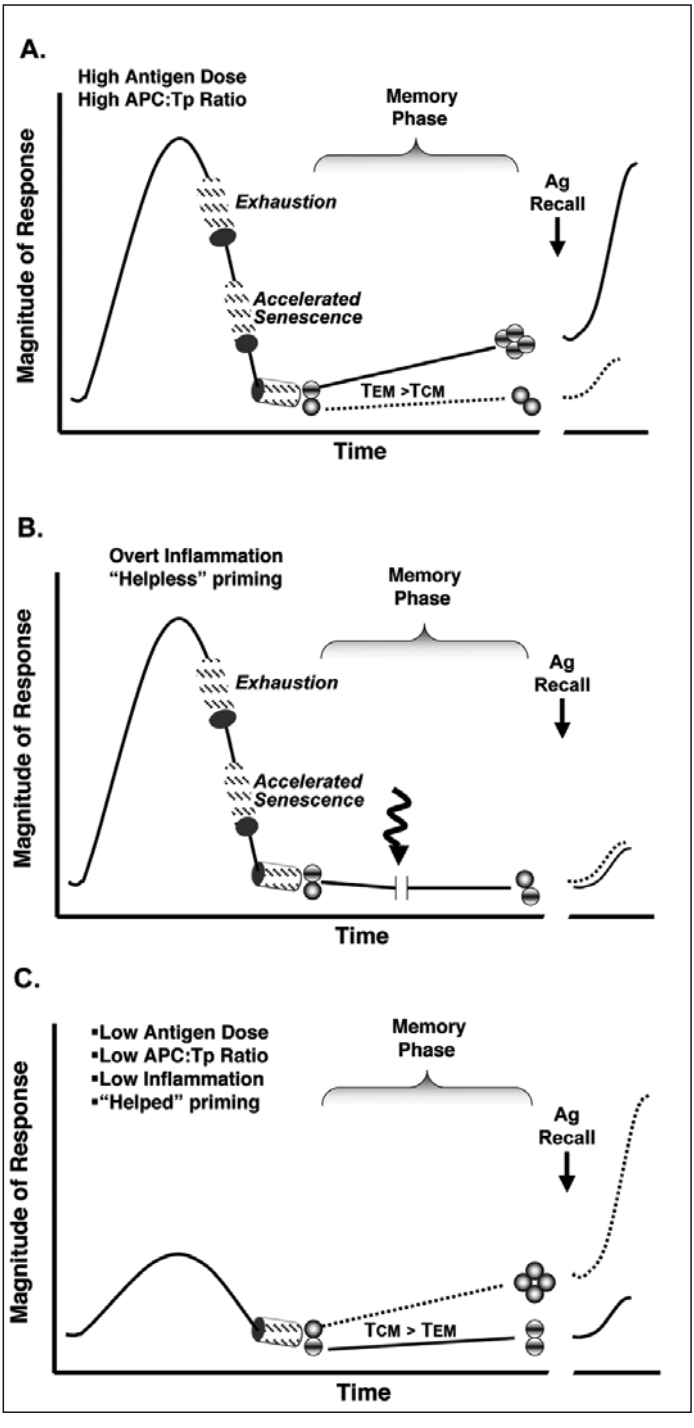

Figure 3. Figure legend viewed on next page.

Figure 3, continued from previous page. Possible scenarios in the generation of memory T cells after vaccination of the immunologically inexperienced individual. B) The expansion phase of the primary T-cell response occurs in the presence of inflammation. During the contraction phase activated effector T cells go through two checkpoints: exhaustion and acceleration of replicative senescence, both set in motion by inflammation-driven cell hyperactivation. T cells that survive the toll of these two checkpoints enter the "germinative hub" where memory cells are generated and lineage differentiation imprinted. Under conditions of high inflammation few memory T cells survive and enter the "germinative hub" and consequently only few T cells will be carried through the memory maintenance phase. Upon Ag recall the expansion of these few memory T cells will be limited. In the case of "helpless" priming the generation of memory T cells will be hindered during both the programming and the maintenance phase. Upon antigen recall only a limited expansion will occur with most cells undergoing TRAIL-mediated apoptosis. A) The expansion phase of the T-cell response occurs in the presence of high antigen (Ag) dose and/or high antigen presenting cell (APC):T-cell precursors (Tp) ratio. Again in this case activated effector T cells go through two checkpoints: exhaustion and acceleration of replicative senescence, both set in motion by high antigen stimulation. T cells that survive the toll of these two checkpoints enter the "germinative hub" where memory cell lineage differentiation is imprinted with a selective advantage for T_{EM} over T_{CM} cells. Upon antigen recall T_{EM} cells are expanded while T_{CM} cells expand proportionally much less. C) The expansion phase of the primary T-cell response occurs at low antigen dose, low APC:Tp ratio, in the absence of inflammation and with T-cell help. In this case activated effector T cells do not go through the exhaustion and acceleration of replicative senescence checkpoints. T cells enter the "germinative hub" where memory cell lineage differentiation is imprinted with a selective advantage for T_{CM} over T_{EM} cells. Upon antigen recall T_{CM} cells expand while T_{EM} cells expand proportionally much less. Reprinted from: Zanetti M, Franchini G. Trends Immunol 2006; 27:511-517.[31]with permission from Elsevier.

Box 2. Precis on principles for the induction of protective memory T-cell responses by vaccination in the immunologically-inexperienced individual

	Principles	***Effects on Memory T Cells***	***Refs***
1	Priming with low antigen dose; prolonged antigen presentation in vivo	• Higher expansion of resting memory T cells upon antigen recall	30,72,87
		• Lineage generation commitment in favor of T_{CM} cells over T_{EM} • Likely to confer protection against exhaustion and reduce the toll of accelerated senescence	59,88,89
2	Priming under the umbrella of CD4 T cell help	• Th cell help increases degree of protection • Favors T_{CM} cell differentiation(?)	43,47
3	Priming avoiding excessive inflammation (TLR agonists, viral vectors)	• Reduces activation of the APC limiting conditions that favor effector generation and T_{EM} cell lineage differentiation	63
		• Bypass restriction of memory T-cell expansion upon antigen recall	26
		• Decelerates replicative senescence • Regulation of T-bet that in turn regulates the memory cell potential	76

necessary for protective responses arguing for a positive effect on lineage differentiation. Finally, the fate of memory T-cell generation is inversely proportional to the degree of inflammation at the time of priming. Overt inflammation has been shown to play adversely on the induction of memory T cells at different levels. These include restriction of the expansion upon recall infection,[26] bias in favor of T_{EM} cell generation[63], and modulation of the levels of the transcription factor T-bet in activated T cells which in turn regulates the memory cell potential.[76]

Collectively, this set of working principles are paradoxically at variance with commonly used vaccination practices relying on high antigen dose and local inflammation by either potent immunological adjuvants (e.g., TLR agonist) or viral vectors to elicit primary T-cell responses that can be easily detected in the aftermath of priming. These practices are owed mainly to a tradition that did not, nor could have had, an understanding of the correlation between T-cell immunity and protection that exists today. We are not surprised that contemporary trial with viral vector vaccines[77,78] have not met with the desired success.

The inference to be drawn is that the recipe for vaccination of the immunologically inexperienced individual to induce protective T-cell responses should follow the simple principle that more is not better and that, paradoxically, the survival of the fittest in the context of vaccination is achieved with maximal economy of means.[31] These concepts are illustrated in Figure 3.

Principles for Reprogramming Protective T-Cell Responses by Vaccination in the Immunologically Experienced Individual

Given the incidence of certain chronic viral disease in the population and their morbidity, the prospect of an effective post-infection vaccination strategy is as necessary as a preventive one. Here again in collaboration with Dr. Franchini we performed studies in Rhesus macaques. Through retrieval and analysis of archival samples we demonstrated that vaccination of already infected macaques yield a control of viremia that correlates with the induction and persistence of CD8 T_{CM} cells provided that macaques were subject to a cycle of anti-retroviral therapy (ART) prior to vaccination.

Briefly, we analyzed 24 macaques divided into three groups. A group of macaques were treated with ART and mock vaccinated with the highly attenuated vaccinia virus strain, NYVAC, whereas the remaining macaques were vaccinated with a NYVAC-SIV construct in the presence or absence of ART. The relative frequency of Gag181-189 CM9 tetramer-positive T_{CM} and T_{EM} cells before, during and after all treatments was measured over a period of months. We found that immunization with NYVAC-SIV of ART-treated macaques expanded approximately eight-fold the mean frequency of the Gag181-189 tetramer-positive cells when compared to unvaccinated controls. We also found that in the absence of ART, NYVAC-SIV was unable to expand these responses. A distinction of Gag181-189 tetramer-positive into cells with T_{CM} or T_{EM} characteristics demonstrated a significant increase in the frequency of T_{CM} cells in macaques vaccinated while on ART.[73] Interestingly, a significant negative correlation between viremia and CD8 T_{CM} cells persisted following ART cessation. Thus, while a correlation between protection from disease and T_{CM} cells was again evidenced, the value of these new studies is that the induction of central memory CD8 T cells can be attained in the infected individual if the antigen load is first abated by anti-retroviral therapy. These concepts are illustrated in Figure 4 where vaccination of the highly viremic individual and the individual in which viremia has been abated by anti-retroviral therapy are placed in relation to exhaustion, accelerated senescence and memory T-cell lineage differentiation.

The Role of Local Immunity in Protection by Memory CD8 T Cells

The defense against many pathogens, and in all likelihood cancer cells, begins in the lymph nodes draining the portal of entry (microbial pathogens) or the site of pathology (cancer cells). Excluded from this scenario is the response to pathogens transmitted by arthropods, which generally cause the systemic spread of the pathogen with involvement of the spleen as a secondary lymphoid site to originate a primary immune response. This category comprises flea-transmitted

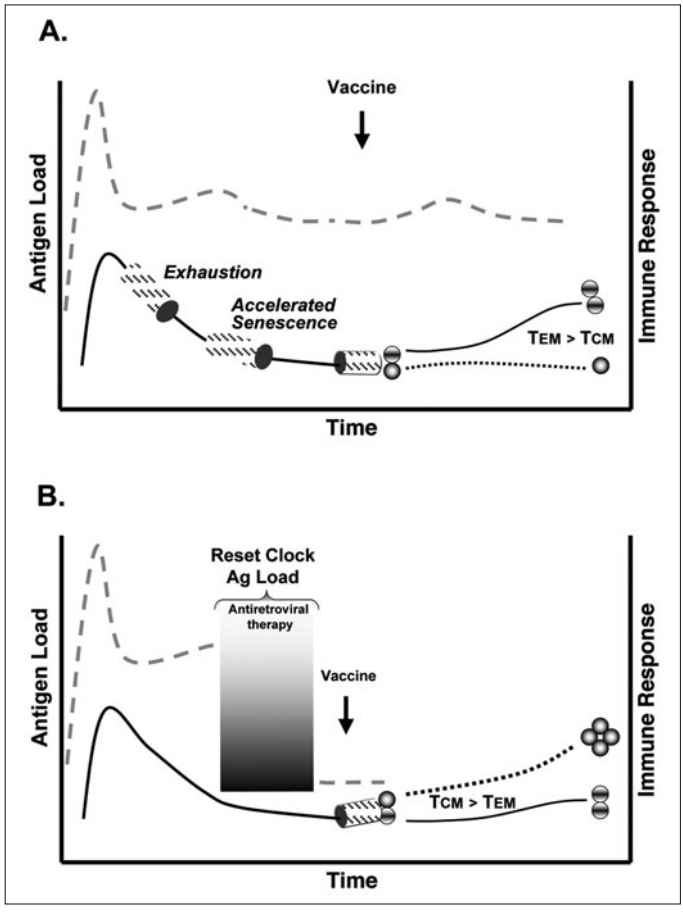

Figure 4. Possible scenarios in the generation of memory T cells after vaccination of the immunologically experienced, infected individual (post-infection vaccination). A) Conditions of high antigen load and inflammation limit the immune response that follows infection leading to negative regulation at the exhaustion and acceleration of replicative senescence checkpoints. Upon vaccination T cells enter the "germinative hub" which imprints memory cell lineage differentiation with a selective advantage for T_{EM} over T_{CM} cells. Persisting conditions of inflammation and high antigen load hamper the expansion of these memory T cells leading to a limited expansion of T_{EM} cells only. B) Under conditions in which the antigen load is reset, vaccination favors the selective imprinting of T_{CM} over T_{EM} cells in the "germinative hub" and allows their subsequent expansion. Reprinted from: Zanetti M, Franchini G. Trends Immunol 2006; 27:511-517.[31] with permission from Elsevier.

disease like plague, mosquito-transmitted viruses such as equine encephalitis and West Nile viruses, flaviviruses, malaria parasites, Rift Valley fever, and yellow fever viruses.

To exemplify the concept of local immunity as it relates to protective memory T-cell responses we will use as an example influenza virus infection. By tracking peptide/MHC complex-specific DCs in vivo following primary virus infection we determined that antigen presentation is confined to CD8a negative DCs of the draining lymph nodes and is maximal on day 2. We also determined that activation of CD8 T cells (CD69+) correlates with the timing of complex detection and their proliferation (dilution of CFSE) occurs in the draining lymph nodes 3 days after infection.[79]

Table 1. Lack of restimulation by myeloid DCs in lymph nodes draining the airway tract is essential to protection by memory CD8 T cells

Mice	Vaccination	CD62^{hi} CD8 T-Cells Transfer	DC Reconstitution	Lethal Virus Challenge	Survival %
C57Bl/6	+	–	–	+	100
	–	+	–	+	100
RelB (–/–)	+	–	–	+	0
	–	+	–	+	0
	–	+	+	+	100

Peptide/MHC complex-specific DCs are not detected in the lung nor are antigen-specific T cells. Thus, though DCs and macrophages are present at the alveolar surface in the lung, antigen presentation occurs exclusively in the draining lymph nodes as site of immune induction.

As noted above, vaccine-induced memory CD8 T cells (T_{CM}) protect from lethal influenza virus challenge.[71,72] However, the relationship between local immunity and protection, the ultimate important parameter in the control of influenza A virus morbidity and mortality, is not fully understood. To address this issue we performed experiments in a mouse model of DC deficiency. Homozygous *rel*B (–/–) mice possess no LNs and lack functional bone marrow-derived DCs[80] although they possess a population of CD8a$^+$ lymphoid DCs in the spleen.[81] Bone marrow chimeras generated by transferring *rel*B (–/–) bone marrow cells into lethally-irradiated hemizygous (+/-) *rel*B recipients carry the same DC defect as *rel*B (–/–) mice but have a longer life span.[82] In fact, *rel*B (–/–)spleen DCs are unable to prime T cells.[83,84] However, when immunized with transgenic B-lymphocytes such as APCs *rel*B (–/–) bone marrow chimeric mice mount both CD4 and CD8 T-cell responses demonstrating that T-cell priming in the absence of myeloid DCs is possible.[85] Using this model we then asked the question: "Can memory CD8 T cells protect in the context of DC deficiency?"

Vaccination with transgenic B-lymphocyte failed to protect mice carrying bone marrow-derived DCs with *rel*B deficiency[86] (Table 1). We reasoned that lack of protection in vaccinated *rel*B (–/–) mice could result from the defective reactivation of memory CD8 T cells in the LNs draining the lung rather than an impaired T-cell response. The explanation seemed plausible and testable only requiring the adoptive transfer of immune CD62L^{hi} CD8 T cells generated in C57BL/6 mice into *rel*B (–/–) mice. As expected the transfer of memory CD8 T cells fully protected C57BL/6 mice but not *rel*B (–/–) mice (Table 1). Protection was, nonetheless, restored in full by injecting 5x10^5 C57BL/6 bone marrow-derived DCs pulsed with the influenza $NP_{366-374}$ peptide 24 hours after memory T-cell transfer and 24 hours prior to lethal virus challenge (Table 1). The combination of these two results strongly implicated a defect in the function of memory T cells once in the *rel*B deficient environment which could be due either to poor trafficking by the adoptively transferred memory T cells or to inadequate antigen presentation by the *rel*B (–/–) DCs in the lymph nodes draining the airway tract. The latter defect became apparent when antigen presentation by *rel*B (–/–) DCs isolated from the draining lymph nodes 24-48 hours after intranasal challenge with the A/HKx31-OVA influenza virus was analyzed with a monoclonal antibody specific for the SIINFEKL peptide complexed with the K^b molecule. While marked antigen presentation was observed by DCs in C57BL/6 mice we found no presentation by DCs in *rel*B (–/–) mice (Fig. 5). Together these results raise an important point: memory CD8 T cells must be reactivated by DCs in the draining LNs in order to mediate protection. Thus, systemic and local immunity need to complement each other, to establish a communication between site of infection and pathology and the site of immune induction by natural infection (Fig. 6).

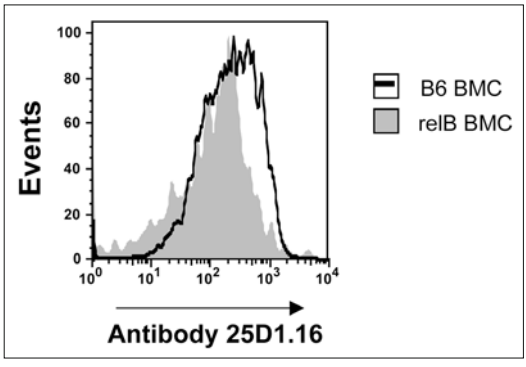

Figure 5. Antigen presentation by DCs in lymph nodes draining the airway tract. Memory CD8 TCR transgenic OT-I cells were adoptively-transferred into C57BL/6 mice or *rel*B (–/–) BMC and mice were challenged 24 hours later with a sublethal dose of A/HKx31-OVA influenza virus. Twenty four hours after infection, draining LNs and spleens were removed, digested and stained for DCs (CD11c+) and presentation of SIINFEKL/K^b (clone 25.D-1.16). CD11c+ cells presenting the SIINFEKL peptide complex with the K^b molecule are shown. (Reproduced from: Castiglioni P et al. J Immunol 2008; 180:4956-4964;[86] with permission.)

Conclusion

The value of the hypothesis that central memory CD8 T cells are operative in protection against disease caused by viruses is in itself an important step forward to clarify the immunodynamics of protective responses after vaccination. The synthesis attempted herein provides for direction for observation and for experimentation of theoretical and practical importance. Irrespective of the type of vaccine, the principles discussed lay the foundation for a rational understanding of the immunodynamics of the correlate for protection by T cells.

Our purpose in presenting the forgoing ideas was to try to view the problem through a wide-angle lens to ultimately focus on a set of general principles that take into account the dynamics of the T-cell

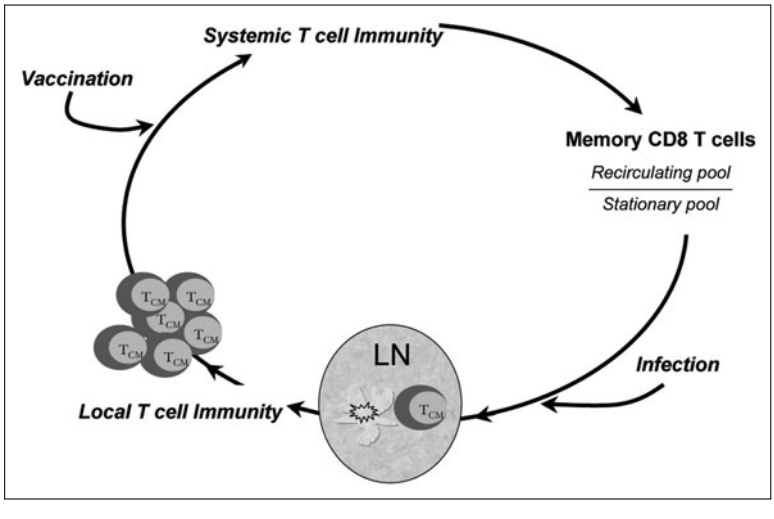

Figure 6. Relation between vaccination-induced systemic T-cell immunity and the role of local immunity in protective T-cell responses. A general idea based on the interaction between systemic and local immunity based on the influenza virus system.

response, the effect of antigen dose and antigen presentation, and the characteristics surrounding the initial contact with antigen including inflammation and over- activation.

The notion that vaccine induced activation of T cells can protect from disease induced by viruses is premised on a still imperfect understanding of lineage differentiation of memory T cells into effector and central type, their replicative life, and the dynamics of telomere attrition versus function development. Taken together, and in light of experimental evidence in patients with chronic viral infections, it appears as if memory T-cell lineage differentiation can be imprinted not only in the immunologically inexperienced individual but also in the already infected, immunologically experienced individual, possibly at various stages of disease, provided that the foregoing principles are taken into consideration. Thus while single ideas could be erroneously equated to vaccine formulation, we have drawn attention to a set of events establishing the relationships among them into a coherent scenario. By integrating information from experimental evidence and extrapolating it into a simple theoretical framework we believe a new basis for vaccination design and implementation may be set.

Notwithstanding the fact that a great deal still needs to be learned to further clarify our thoughts about this issue we begin to see how significant progress has and will be made departing from traditional vaccines that control infection by the induction of antibodies. The effectiveness of these vaccines has been a testimony to the success of medicine in the 20th century. It is our view that progress in understanding the relationship between vaccination and protection by T cells along the lines discussed in this paper will be critical for the development of safe and effective strategies of vaccination to control disease in the future. The next new frontier may also require to de-convolute the genetic program and the transcriptional events that regulate lineage differentiation of memory T cells so that the induction of protective responses against diseases for which vaccines do not yet exist can be facilitated.

Acknowledgement

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Chapter 10

Memory T Cells in Rhesus Macaques

Monica Vaccari and Genoveffa Franchini*

Abstract

The Rhesus macaque (*Macaca mulatta*) is one of the best studied species of Old World monkeys. DNA sequencing of the entire Rhesus macaque genome, completed in 2007, has demonstrated that humans and macaques share about 93% of their nucleotide sequence. Rhesus macaques have been widely used for medical research including drug testing, neurology, behavioral and cognitive science, reproduction, xenotransplantation and genetics. Because of the Rhesus macaque's sensitivity to bacteria, parasites and viruses that cause similar disease in humans, these animals represent an excellent model to study infectious diseases. The recent pandemic of HIV and the discovery of SIV, a lentivirus genetically related to HIV Type 1 that causes AIDS in Rhesus macaques, have prompted the development of reagents that can be used to study innate and adaptive immune responses in macaques at the single cell level. This review will focus on the distribution of memory cells in the different immunologic compartments of Rhesus macaques. In addition, the strategies available to manipulate memory cells in Rhesus macaques to understand their trafficking and function will be discussed. Emphasis is placed on studies of memory cells in macaques infected with SIV because many studies are available. Lastly, we highlight the usefulness of the Rhesus macaque model in studies related to the aging of the immune system.

Introduction

The ability to maintain memory after encounter with an antigen is one of the central features of the immune system. The memory T-cell pool functions as a dynamic repository of antigen-experienced T-lymphocytes that accumulate over the lifetime of a host. While naïve T cells even with optimal T-cell receptor (TCR) stimulation and costimulation are largely incapable of immediate synthesis of certain effector cytokines such as interferons (IFNs), memory T cells acquire this capability upon receiving appropriate differentiation signals during the transition of T cell from naive to memory state.[1,2]

The most advanced discoveries about the induction, development, maintenance and function of the T-cell memory pool have been derived from studies in mice. However, because there are substantial differences in the life span and the immune system of mice and humans,[3] studies in non-human primates (NHP) can provide information on the applicability to humans from concepts derived in murine models.

Most NHP used in the United States and European Union for research on HIV Type 1 and 2 are Old World monkeys that diverged from humans approximately 30 million years ago.[4] These animals are not inbred and are naturally infected by viral pathogens highly related to human CMV, EBV, HTLVI and II, HHV8 and HPV.

Three macaque species account for 79% of all NHP used in research in the UK and 63% of all federally funded research grants for projects using primates in the U.S.[5] The Rhesus macaque (RM) model is the best model for studying the pathogenesis of SIV and for evaluating vaccines for HIV Type 1. In fact, SIV inoculation in RM causes immunodeficiency disease that is similar to that

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Memory T Cells, edited by Maurizio Zanetti and Stephen P. Schoenberger.

Table 1. Commercial antibodies that cross-react with rhesus macaques cells

Naïve and Memory T Cells	Beckton Dickinson	Beckman Coulter	Invitrogen	Milteny	eBioscence
CD3	SP34	10D12	FN18,Cris-7		
CD4	Leu-3A, L200	13B8	S3.4		OKT4
CD8	RPA-T8, SK1	B9.11	3B5	2B5	OKT8, RPA-T8
	17d8, G42-8		143-44	Bw135/80	HIT8a
CD8β	2ST8.5H7				
CD11a	HI111	25.3			HI100
CD28	CD28.2, L293	CD28.2	15 E8	15 E8	CD28.2
CD45RA	5H9, L48	2H4, ALB11	MEM-56	T6D11	
CD45RO*					
CD62L	SK11				
CD95	DX2		DX2	DX2	DX2
T Regulatory Cells					
CD25	M-A251	1HT44H3	3G10	4.00E + 03	BC96
CTLA4	BNI3.1	BNI3			14D3
FoxP3				3G3	PCH101
Cytokines					
IL-2	MQ1-17H12	N7.48A	MQ1-17H12	N7.48A	MQ1-17H12
IL-17**					
IFNα	B27, 4SB3	4S.B3	B27, MD1	45-15	
TNFβ***	359-81-11				
Chemokines and Proliferation					
CCR5****	3A9				
CCR7					3D12
ki67	B56				

*Dako, clone OPD4. **R & D System KIT. ***very weak staining. ****R & D clone CTC5.

observed in HIV infected individuals, both from a virological (tropism and kinetic of expansion of the virus, establishment of reservoir) as well an immunological prospective.[6-9] Because of these studies, a large body of knowledge has been accumulated on the MHC-I and II diversity in these species. Also, several phenotypic markers that define innate and adaptive immune cells are highly conserved between humans and Old World NHP (see Table 1).[10]

Memory T Cells in Rhesus Macaques

Phenotypic Characterization of T-Cell Memory Subsets

The memory T-cell population in RM has been phenotypically characterized in the blood lymphoid and mucosal tissues of neonate and adult animals.[10] Markers that define human T cells

subsets have been studied in macaque's cells using cross-reactive monoclonal antibodies (mAbs) to human lymphocyte surface antigens in flow cytometric analysis (Table 1).[1,2,10-12] This method allows simultaneous multi-parametric analysis of the physical and functional characteristics of single cells and both the enumeration and isolation of sorted purified populations.

The optimal separation of memory T cells from the antigen inexperienced naïve T-cell population can be obtained using a pool of mononuclear antibodies that react with different markers simultaneously expressed on the surface of these cells. One study in particular has shown optimal separation of Rhesus macaque CD4 naïve and memory T cells combining markers for the Fas receptor CD95, the costimulator molecule CD28 and β7 integrin. CD8 memory T cells are also characterized using CD28 and CD95 along with the marker for the lymphocyte function-associated antigen 1, or CD11a, instead of β7 integrin.[10] Of note, in RM, the transmembrane tyrosine phosphate known as CD45RA, a common marker for human T cells, is highly expressed by both naive $CD4^+$ and $CD8^+$ T cells. While CD95 along with CD45RA efficiently separates naïve T cells from the rest of the lymphocytes, the remaining population shows great phenotypic heterogeneity, suggesting the presence of different subsets within the memory pool.

Central and Effector Memory T Cells

Memory T-lymphocytes contain distinct populations of central memory (T_{CM}) and effector memory (T_{EM}) cells characterized by distinct homing capacity and effector functions.[13-15] In humans, T_{CM} express lymph node homing receptors (CD62L and CCR7), whereas T_{EM} are mainly located at the effector sites[16] and they express β_1 and β_2 integrins, chemokines such as CCR1, CCR3 and CCR5 and homing receptors such as CD103 and CLA.[17] In mice and humans, T_{CM} differentiate into effector cells upon secondary stimulation while T_{EM} convert to T_{CM} following antigen clearance.[13,18] T_{CM} are the main source of IL-2, a cytokine that induces proliferation of T-lymphocytes, thus displaying greater proliferative potential compared to effector memory T cells. Rhesus macaque's T_{CM} and T_{EM} have been characterized using mAbs to CD28 costimulatory molecule and CD95 Fas ligand[10,19] (Fig. 1). Both the $CD4^+$ and $CD8^+$ T_{EM} lineages express CD95 and low levels of CCR7, a chemokine that controls the migration of memory T cells to the lymph nodes and they also lack CD28 ($CD95^+/CD28^-/CCR7^{lo}$). T_{CM} are also $CD95^+$, but they express high levels of both CD28 and CCR7 ($CD95^+/CD28^+/CCR7^+$). The CD45RA or the CD62L markers, commonly used in humans and in mice to define one or the other subset, have been less extensively used to characterize T_{CM} and T_{EM} in RM. CD45RA marker in combination with CD28 has been used to characterize effector memory ($CD28^-/CD45RA^-$) from terminally differentiated effectors ($CD28^-/CD45RA^+$) $CD8^+$ T cells, cells that have reached the last stage of their differentiation path, thus lacking proliferative potential and expressing high levels of pro apoptotic markers.[20]

A subset of memory T-lymphocytes that co-expresses CD4 and CD8 has also been identified in RM. This subset expresses high levels of CD4 and low levels of CD8α markers ($CD4^{hi}/CD8\alpha^{lo}$) and based on analysis of CD28 and CD95 expression, the majority (80%) of $CD4^{hi}$ $CD8\alpha^{lo}$ lymphocytes display an effector memory phenotype ($CD28^-/CD95^+$). Only a minor fraction of double positive T cells are central memory. This T-Cell subset is particularly abundant in the intestinal lamina propria where it is capable of producing high levels of cytokines and chemokines and relatively high levels of granzyme B.[21,22]

T Regulatory Cells and Th17

Heterogeneity is a hallmark of antigen-specific T cells.[23-27] Upon antigen stimulation, $CD4^+$ T cells can differentiate into different types of effectors cells: T helper cell 1 and T helper cell 2 (Th1 and Th2) represent well know forms of polarized $CD4^+$ T-cell responses. Th1 produce IL-2, IFN-γ and TNF-α, activating phagocitic cells and $CD8^+$ T cells, thus promoting cell mediated immunity and cytotoxic responses[25,26]; Th2 cells produce IL-4, IL-5, IL-9 and IL-13, inducing B-cells to produce immunoglobulin IgG1 and IgE. More recently, two different subsets of T cells have been found: under certain stimulatory conditions and depending on the homing tissues, T cells can differentiate in regulatory T cells (Tregs)[28-32] or IL-17 producing cells (Th17).

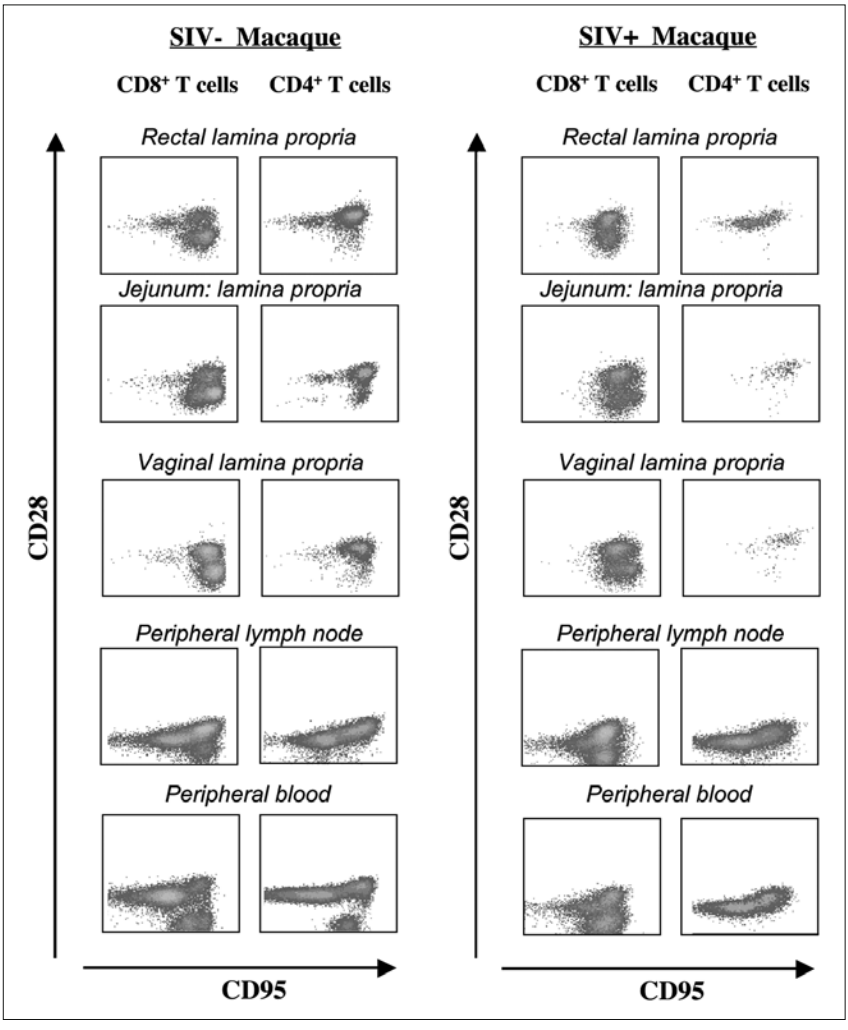

Figure 1. Flow cytometric analysis of memory T-cell subsets in healthy and infected macaques. Raw data from one naïve noninfected animal (left panel) and one SIV infected animal (right panel), during the acute phase of infection.

T regulatory cells are subsets of $CD4^+$ and $CD8^+$ T cells that control immune responses maintaining the balance between immunity and tolerance.[28,29] Regulatory T cells expressing CD4 have been most extensively studied in mice and humans and more recently in Rhesus macaques.[30,31] Tregs are heterogeneous and can be divided into two subsets, naturally occurring, thymic-derived Tregs, that constitute 5-10% of the total peripheral $CD4^+$ T cells in mice and humans[31] and adaptive-Tregs.[32]

Naturally derived Tregs express the interleukin-2 receptor alpha chain CD25[33,34] and they constitutively express several other activation markers, such as the glucocorticoid induced tumor necrosis factor (TNF) receptor-related protein (GITR), OX40 (CD134), L-selectin (CD62L) and the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4 or CD152).[35,36] However, none of these markers exclusively identifies Tregs as they can also be expressed on activated T-cell subsets.

Forkhead box P3 (FOXP3) is an important transcription factor for the development and functionality of Tregs and it is also a more selective intracellular marker to define this population.[37-39] Loss of function mutations in FOXP3, both in mice and men, results in the absence of Tregs, leading to a phenotype with severe autoimmune disorders known as scurfy mice and IPEX (immunedysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) in men.[40,41]Also, the Type I glycoprotein CD127, that is the receptor for IL-7, is down-regulated on Tregs.[42] This population originates in the thymus upon strong antigenic stimulation that requires different signals, through the engagement of the T cells receptor (TCR) and co stimulation molecules, such as CD28. Once activated, Tregs inhibit proliferation of T cells primarily through contact dependent mechanisms. However, different studies have shown that this subset can also function in a cytokine dependent way, through the production of IL-10 and transforming growth factor-β(TGF-β).[43,44]

The origin of the 'adaptive' Treg population is less clear: some studies have proposed that this subset is generated in the periphery from the pool of $CD4^+$ $CD45RO^+$ $CD25^-$ $FOXP3^-$ memory T cells; others suggest that it originates in the thymus and then further differentiates as a consequence of exposure to antigens in distinct immunological contexts.[32] Adaptive Tregs express variable levels of CD25, depending on the disease setting or the site of regulatory activity[45] and they function in vivo in a cytokine dependent manner.[46-48] What remains unclear is the antigen specificity of the adaptive Tregs cells.[49,50]

The suppressive role of Tregs has been studied in the context of many autoimmune diseases as well as cancer[51-53] and in HIV.[54-58] The hypothesis that Tregs may play a role in HIV infection has been tested in the SIV non-human primate model of AIDS. Tregs can be characterized with the same markers used in humans. Ex vivo studies have shown that depletion of $CD25^+$ cells from human PBMC and monkey's peripheral lymph nodes leads to a significant enhancement of $CD4^+$ and $CD8^+$ T-cell responses to select pools of HIV and SIV peptides.[55,59] These data indicate that $CD25^+$ Tregs exert similar functions in humans and Rhesus macaques and suggest their role in decreasing HIV and SIV specific immune responses. In addition, longitudinal studies on SIV-infected Rhesus macaques have revealed a transient increase in the frequency of Tregs from baseline values following acute infection. Also, during chronic infection T regulatory cells accumulate in tissues of infected macaques, especially in the spleen and in the gut, while the frequency of this population decreases in periphery.[56,58] The accumulation of T regulatory cells at these sites has been correlated with disease progression.[56,58]

More recently, another subset of $CD4^+$ T cells has been identified. These activated $CD4^+$ T cells reside mostly in the gut and at the mucosal sites, where they are able to produce IL-17, a cytokine important in the host defense against extracellular bacteria (Th17).[60-66] While the induction and function of CD4 T helper Type 1 and 2 are clear, the full spectrum of function of this subset has not been defined. Together with IL-17, Th17 cells produce IL-22, a cytokine that induces production of antibacterial defensins.[63] In Rhesus macaques, this subset is mainly present in the lamina propria of the colon, the jejunum, ileum and the rectum, and less represented in blood, lymph nodes and spleen.[64] Th17 can be identified upon in vitro stimulation with CD3 or phorbol myristate acetate (PMA) and ionomicin.

When stimulated in vitro, Th17 cells express CCR5 and CD95 and can be positive or negative for CD27. The frequency of CD4-producing IL-17 significantly declines in the gastrointestinal (GI) tract during the early phase of infection with HIV/SIV. This loss may explain the chronic enteropathy in HIV infection.[64-66]

Tissue Distribution of T-Cell Memory Subsets in Rhesus Macaques

Many of the cellular and molecular processes involved in forming and maintaining immunological memory are still unknown. Studies in mice have elucidated the distribution of memory T cells in different tissues. In vitro and in vivo imaging in mice have demonstrated that antigen-stimulated memory T cells migrate from the lymphoid tissues to nonlymphoid tissues where they form the first line of defense against re-encountered pathogens.[67,68] Memory T cells that remain in the lymphoid tissues constitute a reservoir that can be mobilized again when

necessary. The physiological distribution of memory T cells in humans is less known, due to the difficulty of sampling healthy individuals. Therefore, the Rhesus macaque model serves as a valuable tool to study the immunobiology of different lymphoid and nonlymphoid compartments in a model that closely relates to humans.

In macaques, naïve and effector/memory T cells express different trafficking ligands and receptors and consequently have distinct patterns of migration.[69,70] Memory T cells are localized in lymphoid tissues and have the ability to traffic to various extra lymphoid tissues of the body, also called effector sites.[10] The anatomic location plays an inductive role in the $CD8^+$ T cells memory differentiation program. In fact, it has been shown in mice, that virus-specific intraepithelial lymphocytes in gut resemble neither central nor effector memory $CD8^+$ T cells isolated from spleen or blood, suggesting that memory $CD8^+$ T cells may change phenotype upon changing location.[71,72]

Peripheral Blood and Lymph Nodes

Naïve and memory T cells coexist in the peripheral blood of Rhesus macaques, as well as in humans. The CD4 memory population in the periphery is mainly CD28 positive (T_{CM}), while both central ($CD28^+/CD95^+$) and effector memory ($CD28^-/CD95^+$) $CD8^+$ T cells coexist in this compartment (Fig. 1).[10]

The development of T-cell immune responses starts with delivery of an antigen (Ag) from an exposed tissue site to the draining lymph node. Naive T cells that constantly recirculate from the blood to the lymph nodes are activated and differentiate into effector and memory T cells. Following differentiation T cells express new cell surface molecules that allow them to home to nonlymphoid tissues.[73-75] These activated T cells express effector cytokines and are unable to return to the draining lymph nodes or to the pool of circulating lymphocytes.[76] Memory $CD8^+$ T cells present in the lymph nodes are important as a first line of defense to pathogenesis as they curb the spread of pathogens from the lymph node to vital organs at very early stages of infection.[77] Peripheral lymph nodes of RM contain both $CD4^+$ and $CD8^+$ T cells and the main subsets are naïve and central memory T cells ($CD28^+/CD95^+$)(Fig.1).[10]

The Gastrointestinal Tract

The gastrointestinal tract is a prominent part of the immune system and is enriched with memory T cells that predominate in the intestinal lamina propria (lamina propria lymphocytes, LPL) and in the epithelium (intraepithelial lymphocytes, IEL).[78] The gut associated lymphoid tissues (GALT) of RM has been extensively studied in the context of SIV infection and during disease progression to simian AIDS. In fact, the GALT is the primary site of replication for HIV/SIV. The remarkable similarity in the composition of the GALT between humans and Rhesus macaques has justified the use of these animals as model for human AIDS.[79-83]

The intra-epithelial lymphocytes (IELs) in the gut of RM are predominantly $CD8^+$ (63-80%) and contain very few $CD4^+$ T cells. $CD8^+$ T cells present a memory phenotype and express the $\alpha E\beta 7$, an integrin that mediate T-cell adhesion to epithelial cells through its binding to E-cadherin.[79,84,85] Lamina propria lymphocytes (LPLs) are a mixed population of $CD4^+$ and $CD8^+$ T-lymphocytes, with a CD4:CD8 ratio that range from 0.74 to 1.3. Memory phenotypes are present at this site (Fig. 1).[79] Both $CD8^+$ and $CD4^+$ T cells express low levels of $\alpha E\beta 7$, are mainly positive for the CD95 marker and the CD8 memory population expresses beta7 integrin. The memory pool contains $CD4^+$ and $CD8^+$ T_{CM} and T_{EM} subsets, as indicated by the presence of positive and negative CD28 and CD95 positive T-lymphocytes (Fig. 1).[10]

In SIV infected macaques, as early as a few weeks from infection with a CCR5 tropic viral strain of SIV, $CD4^+$ T cells in the LP decrease by 50-70% compared to uninfected controls (Fig. 1). The main population that is targeted and killed at this site is activated $CD4^+$ T cells expressing the homing marker $CCR5^+$, which are numerous in the LP but scarce in the periphery.[78-82] A near normal level of $CD4^+$ T cells is maintained in lymph nodes and blood.[81] The CCR5 coreceptor is selectively expressed at effector site on effector memory T cells that are negative for CCR7.[86,87] All $CD4^+$ memory T cells are not equally susceptible to this acute destruction. In SIV infected RMs, the

remaining $CD4^+$ T-cell population is predominantly $CCR5^-$ T_{CM} cells. This population undergoes a substantial increase in proliferative activity that initially provides sufficient production of T_{EM} cell in the effector sites to maintain clinical immune competence.[88] During the chronic phase of infection the frequency of $CD4^+$ T_{EM} cell decreases in the extra-lymphoid immune effector sites of the body, both in humans and RM.[89-90]

The Lung

Similar to the gut, the RM's lamina propria of the bronchoalveolar compartment is composed of memory T cells that express CD95, whereas the frequency of T-lymphocytes that express β7 integrin is lower than in the gut. The central memory pool is the main CD4 subset present in the lung, whereas both T_{CM} and T_{EM} CD8 are represented in this compartment.[10]

The Vaginal Mucosa

The vaginal mucosa of normal juvenile and adult female Rhesus macaques have been examined by flow cytometric analysis and multicolor immunohistochemistry, a process that visualizes antibody-antigen interaction on tissue sections.[91-95] The objective of these studies was to characterize the vaginal mucosa as a primary site for HIV transmission and the role of mucosal immune responses in the vagina and cervix in protection from the virus. Lymphocytes of the vaginal mucosa are localized within the epithelial layer and in the lamina propria. Vaginal lamina propria of macaques contains 55 to 65% $CD8^+$ T cells and 28 to 34% $CD4^+$ T cells, while the majority of intra-epithelial cells are $CD8^+$ T cells (75 to 85%).[91] 54-67% of the $CD4^+$ T cells in the vaginal mucosa express the activation marker CCR5.[92] This population resides in the lamina propria, whereas essentially no CD4 or CCR5 expression can be detected within the squamous or keratinized layers of the vaginal epithelium. CCR5 expression is higher in the vaginal lamina propria of mature macaques compared to 1-3-year-old juveniles. The vast majority of $CD4^+CCR5^+$ lymphocytes in the vagina also express CD95 and CD28 ($CD95^+/CD28^+$) showing a central memory phenotype (Fig. 1). In addition, cytolytic $CD8^+$ T-cell lines derived from the vaginal epithelium are αEβ7 positive and L-selectin negative.[93,94] The vaginal lamina propria of SIV infected female macaques is depleted of almost 40-60% of $CCR5^+CD4^+$ T cells during the early phase of infection.[94,95]

In Vivo Manipulation of Memory T Cells in Non-Human Primates

Autologous Transfer

T-lymphocyte migratory circuits in humans remain largely unexplored due to the difficulty of performing cell trafficking in normal volunteers. Recently, NHP have been used as a model to study how T-lymphocytes migrate to different compartments. Experiments of autologous transfer of labeled peripheral blood mononuclear cells (PBMCs) have been performed in RM to study the homing of T-lymphocytes to lymphoid and nonlymphoid compartments. This method uses the carboxyfluorescein diacetate succinimidyl ester (CFSE), a fluorescent cell staining dye that is retained by the cell in the cytoplasm, which does not adversely affect cellular functions and can be detected by flow cytometric analysis.[96] Using this technique, Clay and colleagues have shown that, within 48 hours of intravenous transfer of CFSE labeled PBMCs, T-lymphocyte trafficking can be detected to the liver and bone marrow and at a lower level to the thymus and small intestine. The liver contains the highest proportion of stained $CD45RA^-$ T-lymphocytes, consistent with homing of activated/memory T-lymphocytes to this nonlymphoid site.[97,98]

Another recent study established tracking of T cells to various compartments of RM as a preclinical model for the evaluation of T-cell-based immunotherapy.[99] In this study, harvested PBMC were either unstimulated or stimulated with antiCD3/antiCD28, then labeled with CFSE and reinjected intravenously into the donor animals. Blood samples, lymph node biopsies and biopsies from duodenum and rectum were collected at various time points and analyzed by flow cytometric analysis for the presence of the reinjected T cells. The authors showed that non-specific in vitro activation changes the in vivo migratory behavior of T cells. In fact, they observed a preferential

migration of activated $CD8^+$ T cells to the rectum, while non-specifically activated transferred $CD4^+$ T cells were found in much lower frequencies at this site and also in other compartments.

In Vivo Studies of T-Cell Turnover

The rates of lymphocyte turnover during health and disease are poorly characterized. This limits the understanding of diseases like HIV infection[100,101] that lead to increased rates of cellular turnover and ultimately to deterioration of the immune system. Since the NHP is the primary model for HIV infection of humans, techniques have been developed to study T-cell turnover and loss, in healthy and SIV infected macaques. Proliferative activity of T-lymphocytes can be monitored in ex vivo experiments by flow cytometric analysis through the evaluation of Ki67 antigen, a marker of the cell cycle progression.[10] A more advanced technique uses the administration of 5-bromo-2′-deoxyuridine (BrdU), a thymidine analog measuring the rate at which cells become labeled with this DNA precursor during the S phase of the cell cycle. BrdU can be administered to the animals via drinking water. Cells that contain BrdU are then detected ex vivo using fluorochrome-labeled antiBrdU monoclonal antibody by flow cytometric analysis.[102] Various mathematical models have been developed that permit estimates of different parameters from the data generated by this type of experiment.[103,104] One of these models has been used to analyze BrdU labeling curves for a number of different lymphocyte populations in healthy and SIV infected Rhesus macaques.[104] This method has been used by Luka Čičin-Šain and colleagues to test whether naïve T-cell turnover is increased in aged monkeys. In this study, cohorts of adult and old monkeys were pulsed with BrdU on days 0 and 3 and assayed for its incorporation/decay kinetic in blood T cells over time. This technique allows the concomitant detection of BrdU positive cells that proliferated at any time during the days of BrdU administration, of actively proliferating cells ($Ki67^+/BrdU^+$) and of cells that ceased proliferating by the time of detection ($Ki67^-/BrdU^+$).[105]

Kaur and colleagues have studied the perturbations in lymphocyte dynamics in sooty mangabeys, the natural hosts of nonpathogenic simian immunodeficiency virus (SIV) infection during in vivo administration of BrdU.[106] Using the same technique, Picker and colleagues demonstrated that IL-15 dramatically increases in vivo proliferation of RM $CD4^+$ and $CD8^+$ T_{EM} cells, with little effect on the naive or T_{CM} subsets.[107]

Thymectomy

T-cell maturation partially occurs in the thymus, where lymphocyte precursors from the bone-marrow become thymocytes and mature into T cells. Once mature, T cells emigrate from the thymus and constitute the peripheral T-cell repertoire responsible for directing many facets of the adaptive immune system. Loss of the thymus at an early age through genetic mutation or surgical removal results in severe immunodeficiency and a high susceptibility to infection. To study extra thymic T maturation, a method of thymectomy was developed in macaques by Healy and colleagues.[108] The role of the thymus in the pathogenesis of AIDS is a frequently discussed and controversial topic. Therefore, this method has been used to study the role of the thymus in peripheral T-cell homeostasis and to assess the significance of thymic output in SIV infection of RM. By surgical removal of the thymus in juvenile Rhesus macaques, the authors reported that complete abrogation of thymic output in juvenile Rhesus macaques resulted in a faster decay of peripheral $CD4^+$ T cells, but did not cause a substantial shift in $CD45RA^+$ and $CD45RA^-$ populations. In conclusion, thymectomy had very little impact on the peripheral T-cell compartment, both in healthy and in SIV-infected macaques.[109,110]

In Vivo Depletion of T-Cells Subsets

It is difficult to perform studies that assess the role of cell-mediated immune responses during viral infections in humans. Indirect correlations between the frequency of antigen specific $CD4^+$ and $CD8^+$ T cells and virus levels are informative, but they do not directly prove the importance of these responses during the course of infections. Therefore, animal models that permit passive administration of immunoglobulin to naïve hosts have been crucial for demonstrating the contribution of specific components of the immune system in controlling certain infections. Non-human

primates provide valuable animal models for human diseases. A Rhesus monkey model of $CD8^+$ cells depletion using a mouse-human chimeric monoclonal antibody has been developed by Schmitz and colleagues[111] (Table 2). Administration by the intravenous route of this antibody results in nearly total depletion of $CD8^+$ lymphocytes from the blood and lymph nodes for 2-6 weeks, leaving CD4 cell-mediated immune responses and humoral immune responses intact. In vivo CD8 depletion in RM has been used to study the importance of this population during SIV and other infections. Rhesus monkeys were depleted of $CD8^+$ lymphocytes by monoclonal anti-CD8 antibody infusion and then challenged with wild-type measles virus.[112] The $CD8^+$ lymphocyte-depleted animals exhibited a more extensive rash, higher viral loads at the peak of virus replication and a longer duration of viremia than did the control antibody-treated animals, suggesting a central role for $CD8^+$ lymphocytes in the control of measles virus infections. A $CD4^+$ T-cell depleting antibody has first been used in chimpanzee infected with Hepatitis C to demonstrate that memory $CD4^+$ T cells are essential for protection. Indeed, $CD4^+$ T-cell depletion in chimpanzees before re infection impaired the ability of these animals to clear virus despite the presence of functional intra-heapatic $CD8^+$ T cells.[113]

The role of vaccine-induced $CD8^+$, $CD4^+$ T and B cells in protection from monkey pox virus, a virus ortholog of smallpox, has been dissected in Rhesus macaques.[114] Neither $CD4^+$ nor $CD8^+$

Table 2. Antibodies for in vivo depletion assays

AntiAb	Clone/ Commercial Name	Detection mAb	Number of Doses	mg/kg	Route	Ref.
aCD8	cM-T807	DK25	4 doses	1 × 10, 3 × 5	s.c./i.v.	116
		DK25/SK1	3 doses	5	Intravenous	111/112
		DK25	3 doses	1 × 10, 2 × 5	s.c/i.v	114/117
	T87PT3F9*		2/4 doses	2	Intravenous	115
aCD4	OKT4	L200	1 dose	50	Intravenous	114/117
aCD20	Rituxan**	J4.119 (CD19)	4 doses/ every wk	20	Intravenous	114
aCD16	3G8	DJ130C	1 dose	50	Intravenous	119, 120

s.c. subcutaneus. i.v. intravenous. *Coulter. **Genentech and IDEC Pharmaceuticals.

Antibodies for in vivo blocking assays

AntiAb	Clone/ Commercial Name	Detection Ab	Number of Doses	mg/kg	Route	Ref.
aCD40L*			1 dose	20	Intravenous	131
CTLA4 -Ig*			1 dose	20	Intravenous	131
aCTLA4	MDX-10**		2 dose	10	Intravenous	55
			4 dose	10	Intravenous	133

*Baxter Healthcare Corp., Deerfield, Illinois, USA. **Medarex, Inc.

T-cell depletion in Dryvax vaccinated macaques affected vaccine induced protection from the disease. In contrast, antibody-mediated depletion of B cells during immunization abrogated protection and importantly, passive transfer of immunoglobulines from vaccinated individuals restored protection, indicating that protection from smallpox is antibody-mediated.[114]

Different groups have shown that in vivo depletion of $CD8^+$ T cells during SIV infection of RMs results in marked increases in plasma viral load, suggestive of a key role for $CD8^+$ T cells in controlling levels of SIV replication.[115-117] The main limitation of this model is the fact that the antibody used for depletion is not specifically targeting only adaptive $CD8^+$ T-lymphocytes because a high frequency of natural killers (NK) of RMs also express CD8.[118] This leaves an open question about the role of innate responses in the control of SIV replication. Two recent studies have partially ruled out the importance of cytotoxic $CD16^+$ NK cells in controlling AIDS virus replication during primary and chronic infection.[119,120] In fact, transient depletion of NK cells from two Rhesus monkeys chronically infected with simian immunodeficiency virus failed to induce changes in virus replication.

A large body of literature in murine models indicates that CD4 help is not required for a generation of specific $CD8^+$ T cells responses, but is essential to maintain a pool of memory CD8 able to expand after a second encounter with an antigen.[121-123] In vivo CD4 depletion has also been used in macaques as a mean to investigate the importance of helper T cells on the generation and maintenance of SIV specific $CD8^+$ T cells.[117] Depletion of CD4 cells was performed during immunization to decrease the functionality of $CD8^+$ T cells. Treatment with the CD4-depleting antibody resulted in the complete absence of $CD4^+$ T cells from the blood, leaving the frequency of $CD8^+$ T cells and CD20 population intact. The reconstitution of the CD4 population was slow and incomplete, as was previously observed in humans.[124] Vaccinated macaques treated with the CD4-depleting antibody developed less functional $CD8^+$ T cells, resulting in lost control of SIV replication earlier than vaccinated macaque controls.[117] (see Table 2 for details on treatment with depleting antibodies) .

Blocking Antibodies In Vivo

The generation of adaptive immune responses is a highly regulated process that requires the interaction between antigen presenting cells and $CD4^+$ T cells via the major histocompatibility complex (MHC) class 2 and the T-cell receptor (TCR) and also involves numerous costimulatory pathways, aimed to control the balance between immune stimulation and tolerance. One of those pathways involves the binding between the CD40, a protein, expressed by all mature B cells, as well as by dendritic cells, macrophages, fibroblasts, epithelial cells and endothelial cells[125,126] and CD40 ligand (CD40L; also known as CD154), expressed by activated T and B cells and activated platelets. The interaction between CD40 and CD40L promotes both humoral and cell-mediated immune responses and is crucial for the induction of effective adaptive immune and inflammatory responses.[127] Also, the murine CD40L-CD40 interaction between CD4 T helpers and dendritic cells or $CD8^+$ T cells augments the generation of CD8 memory T cells following viral infections.[128]

A second pathway involves the binding of the CTLA4 (Cytotoxic T Lymphocyte Antigen 4), a CD28-family receptor constitutively expressed on regulatory $CD4^+$ T cells, to CD80 and CD86 costimulatory molecule expressed on B cells and dendritic cells. CD28 also binds to the CD80 and the CD86, but CTLA4 has higher affinity than the CD28 and in contrast to CD28 which enhances T-cell function, CTLA4 inhibits T-cell activation.[129]

In mice, the in vivo administration of blocking antibodies to CD40 and to CD28 results in potent and specific immune suppression.[130] In Rhesus macaques, administration of CTLA4 immunoglobuline (Ig) and anti CD40 Ligand prevent renal allograft rejection.[131]

The NHP model has also been used to assess the role of these pathways in the generation of SIV-specific CD4 helper, $CD8^+$ T cells and antibodies.[55,132] Garber and colleagues induced the in vivo blockade of CD28 and CD40 T-cell costimulation pathways with the aim to experimentally induce tolerance to SIV antigens in infected Rhesus macaques. Transient administration of

CTLA4-Ig and anti-CD40L mAb to SIV-infected macaques resulted in dramatic inhibition of the generation of both SIV-specific cellular and humoral immune responses.[132]

The impact of immune activation in SIV infection has been addressed directly by inhibiting CTLA4 during the acute and chronic phase of infection.[55,133] In vivo CTLA4 blockade significantly increased T-cell activation and viral replication in primary SIV infection, particularly at mucosal sites and increased the expression and activity of the indoleamine 2,3- dioxygenase (IDO), an enzyme that converts tryptophan to N-formyl-kynurenine which suppresses T-cell proliferation.[134] Accordingly, protracted anti-CTLA4 treatment of macaques chronically infected with SIV and treated with ART, decreased responsiveness to antiretroviral therapy and abrogated the ability of therapeutic T-cell vaccines to decrease viral replication (see Table 2 for details on treatment with blocking antibodies) .

Differentiation of Memory T-Cells Subsets: Lesson from In Vivo Studies in Non-Human Primates

Studies in humans have implicated a family of cytokines, such as IL-2, IL-7 and IL-15 that use the common γ chain as part of their receptor, as important regulators of peripheral T-cell homeostasis.[135-137] Interleukin-2 (IL-2) exerts a wide spectrum of effects on the immune system and plays crucial roles in regulating both immune activation and homeostasis. IL-2 was identified based on its potent T-cell growth-factor activity and is widely considered to be a key cytokine in T-cell-dependent immune responses both for $CD4^+$ and $CD8^+$ T cells. However, a major nonredundant activity of this cytokine centers on the regulation of T-cell tolerance in the periphery, whereas T-cell immunity to various agents can be readily elicited in the absence of IL-2 in vivo.[138,139]

IL-2 has been evaluated as a therapeutic in the clinical settings of HIV/SIV infection and cancer.[140-143] In vivo administration of IL-2 to Rhesus macaques enhances antigen specific responses. The effects of the administration of IL-2 have been studied as an adjuvant in vaccine strategy for SIV. IL-2 combined with antiretroviral therapy and poxvirus vector based vaccines improves CD4 and CD8 T cells responses and decreases plasma viral load upon ART cessation.[141,142] IL-2 administration ameliorates DNA- based vaccines' efficacy, improving the quantity and the quality of the antigen specific immune responses compared to DNA- based vaccines alone.[142] Villinger and colleagues have shown that following primary immunization to tetanus toxoid (TT) or influenza virus, TT specific $CD4^+$ T cells and influenza matrix protein (Flu-MP) specific CD8 effector responses are enhanced by IL-2 administration, but CD8 specific memory responses are not different from cytokine nontreated monkeys.[143] In that study, the highest levels of primary effector and memory T cells were observed following alternate administration of both IL-2 and IL-15.

Interleukin-7 (IL-7) is a nonredundant cytokine produced by nonlymphoid cells that is essential for T-cell development in humans and mice and B-cell development in mice[144,145] promoting expansion of both thymic and peripheral T-cell populations, the latter including both the $CD4^+$ and $CD8^+$ lineages and both the naive and memory compartments.[146,147] IL-7 contributes to the maintenance of the size and subset composition of the peripheral T-cell pool by providing growth and survival signals through the IL-7 receptor.[148-150] IL-7 modulates memory $CD8^+$ T cells in response to a virus infection.[151]

Administration of recombinant human IL-7 (rhIL-7) and IL-15 to NHP has, in part, elucidated the immunologic effects of these cytokines. Following IL-7 therapy, an increase in the absolute number of naive $CD4^+$ and $CD8^+$ T cells has been observed.[137,152] Upon treatment peripheral T cells up regulate proliferation markers such as Ki67 and BcL2, a survival marker.[153] IL-7 treatment in macaques also induces a transient change in CD11a expression on $CD8^+$ cells with the emergence of a dominant population of $CD11a^{mod}$ cells, suggesting a partial conversion of the naive subset to an activated/memory phenotype. Thus, IL-7 treatment alters peripheral T-cell homeostasis and results in a substantial, but reversible, increase of peripheral blood T-cell number due to faster entry of these cells into cell cycle.[137] Another study from Moniuszko et al,

dissected the effect of IL-7 therapy on different memory T cells subsets of Rhesus macaques.[152] IL-7 induced the acquisition of memory cell markers not only in $CD8^+$ T cells but also in $CD4^+$ T-cell subsets that express both CD28 and CD95 markers and are positive for the proliferation marker Ki67, a protein expressed during all phases of the cell cycle (G1, S, G2 and mitosis), but not in resting cells (G0). Thus, IL-7 increases the frequency of T cells that phenotypically resemble $CD4^+$ T_{CM}. The increase of this memory-like population was dose dependent and occurred in blood as well as secondary lymphoid organs. In addiction, IL-7 increased the ability of $CD4^+$ T_{CM} as well as $CD4^+$ T_{EM} to produce tumor necrosis factor alpha (TNF-α) and to a lesser extent, gamma interferon (IFN-γ) following stimulation with cognate antigen.

Administration of recombinant IL-15 demonstrated that this cytokine play a role in $CD4^+$ T_{EM} cell development and homeostasis in primates.[107,154] IL-15 has proven to be superior to IL-2 in the generation of long-lived antigen specific memory $CD4^+$ and $CD8^+$ T cells in Rhesus macaques.[154] Moreover, IL-15 increases the flux of long-lived $CD4^+$ T cells into extra-lymphoid effector sites. The effect of this cytokine on the $CD8^+$ T-cell population is similar to what has been observed for the CD4 T cells: IL-15 potently induces proliferation of $CD8^+$ T_{EM} cells, with little effect on $CD8^+$ T_{CM} cells.[107] Thus, IL-15, in contrast to both IL-2 and IL-7, selectively expands the $CD4^+$ and $CD8^+$ T_{EM} cell in the extralymphoid tissues.

Several studies have assessed the impact of IL-7 and IL-15 treatment on viral replication alone,[155,156] or in conjunction with vaccines in SIV-infected macaques.[157,158] Beq and colleagues investigated the impact of recombinant simian IL-7 on T-cell renewal in Rhesus macaques chronically infected with SIVmac251 and treated with antiretroviral therapy (ART). This treatment resulted in an increase of the number of circulating $CD4^+$ and $CD8^+$ memory T cells expressing activation and proliferation markers and enhanced thymic function, with no effects on the plasma viral load.[155] Mueller and colleagues have shown that in vivo treatment of acutely SIV infected Rhesus macaques with IL-15 resulted in an increased number of SIV-specific CD8 T cells and NK cells during the peak of viral load. Interestingly, this increase was not maintained during the set point of viremia; on the contrary, at this time animals that had received IL-15 showed an increased viral set point by 3 logs and accelerated development of simian AIDS.[156]

Demberg et al have treated Rhesus macaques with SIV plasmid DNA with or without IL-15 DNA, a multigenic replicating Adenovirus based SIV immunization and two boosts with SIV gp140 and SIV Nef protein.[157] Macaques that were treated with the IL-15 DNA showed a higher peak of anti-Nef antibody titer and expanded SIV-specific $CD8^+$ T cells 2 weeks after the challenge with SIVmac251, compared to the DNA-only group. Although, IL-15 treated macaques did not exhibit lower viral replication and better protection from disease. Finally, our group assessed the impact of recombinant IL-7 and IL-15 treatment on viral replication and the immunogenicity of live poxvirus vaccines in SIVmac251 infected macaques.[158] Neither cytokine augmented the frequency of vaccine-expanded $CD4^+$ or $CD8^+$ memory T cells, clonal recruitment to the SIV-specific $CD8^+$ T-cell pool, or $CD8^+$ T-cell function. Moreover, while vaccination alone transiently decreased the viral set point following antiretroviral therapy suspension, IL-15 induced massive proliferation of $CD4^+$ effector T cells and abrogated the ability of vaccination to decrease set point viremia. In contrast, IL-7 neither augmented nor decreased the vaccine effect and was associated with a decrease in TGF-β expression.

Aging of T Memory Cells

During aging, the immune system undergoes dramatic changes in both structure and function. Macroscopically, the most evident event is thymic involution, which severly diminishes the production of naïve T cells.[159] Consequently, peripheral lymphocyte subset composition is shifted toward the memory phenotype, as an increasing proportion of naïve T cells become exposed to foreign antigens over time. However, the mechanisms linking specific age-related changes in T-cell subset distribution and function to the age-related immunodeficiency are still incompletely understood. Most of the existing data describing the age-related changes in T-cell function come from studies of the rodent's immune system.[160,161] However, even though this

model has been helpful in elucidating some aspect of immune senescence, not all results from rodent models translate to humans, given that these two species diverged approximately 210 million years ago[162,163] and they have a different lifespan (10 folds). Moreover, rodents are held in virtually pathogen-free condition during these studies, thus affecting the number of antigens encountered during their existence and therefore affecting the pool of memory T cells generated and accumulated during life. Non-human primates are better suited for immunogerontologic studies, with direct relevance for human T-cell senescence.[164,165]

With respect to their lifespan, macaques are classified as neonates (first month), juveniles (1-5 years), adult (5-15 years) and old (15-25 years). The memory population in neonates is quite low, but in keeping with the expected result of postnatal Ag exposure, rapidly increases in the first few months of life. Cross-sectional analyses indicate that the average frequency of memory T cells in adult human blood (40-50%) is reached within the first 2-3 years of life in RM.[166,167] After this time, the rise in memory frequencies slows and by middle adulthood (10-15 years), memory frequencies average 70% for $CD4^+$ T cells and 80-90% for $CD8^+$ T cells. These results suggest that RM, especially juvenile animals, may be exposed to more diverse pathogens more frequently than humans.[10]

In vivo and ex vivo studies on cohorts of old RM model have been used as a tool to study the other mechanisms involved in the depletion of naïve T-lymphocytes and in parallel, the accumulation of memory T cells. Through the in vivo administration of BrdU and concomitant analysis of Ki67, a study has shown that in RM, naïve $CD8^+$ and, to a lesser extent, naive $CD4^+$ T cells in old animals exhibit higher proliferation and higher turnover than in young animals.[105] Because the relative size of the naïve subset was also decreased, the authors suggest that elevated turnover comes with a naïve T-cell loss that is equal to or surpasses the elevated proliferation. Also, the authors observed a significant increase in $CD4^+$ T_{CM} cells and $CD8^+$ T_{EM} cells in an aged RM cohort with small naive CD8 pools. Cross-age comparisons revealed no age-related differences in proliferation and turnover of the T_{EM} subsets and an age-related decline in proliferation of T_{CM} CD4 and CD8.

Another study has focused on the effector function of RM $CD8^+$ and $CD4^+$ T cells during senescence.[168] The authors demonstrated that the percentage of cells capable of immediate TNF-α secretion upon T-cell receptor stimulation increases with age among RMs $CD8^+$ T cells, but not among $CD4^+$ T cells. Also, in this study, age-related loss of $CD95^-$ naive cells in RMs did not differ between $CD4^+$ and $CD8^+$ T cells. Therefore, at least among the RMs $CD8^+$ T cells, functional changes within the CD8 memory population appeared to correlate with the aging process better than acquisition of $CD95^+$ $CD28^-$ phenotype.

Conclusion

Humans are much indebted to non-human primates. Their use in research has contributed greatly to scientific discoveries that have improved human health worldwide. Studies in NHPs have made possible the development of protective vaccines against important human pathogens, including Poliovirus and Hepatitis and have been essential in developing a plethora of drugs for treatment of human diseases, such as cancer and AIDS. The genetic and immunological similaries between NHP and humans permit the validation of the relevance of some concepts derived from studies in mice to humans. This is particularly important in studies of the immune system. Hopefully, the use of NHP will also facilitate the full understanding of HIV pathogenesis and guide the development of much needed protective vaccines not only for HIV but also for malaria and tuberculosis.

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Chapter 11

Memory T-Cell Subsets in Parasitic Infections

Sara Colpitts and Phillip Scott*

Abstract

Parasitic infections remain a major health problem throughout the world and unlike many viral or bacterial diseases, there are no vaccines to help control parasitic diseases. While several important advances have been made that will contribute to the development of parasite vaccines, such as cloning of dominant parasite antigens and a better understanding of the effector T-cell subsets needed for immunity, fundamental questions remain about how to induce long-term immunologic memory in vaccines. Here we examine a few of the experimental models that have been used to elucidate the nature of the memory T cells that are generated during parasitic infections. Although significant hurdles remain in the development of parasite vaccines, studies with both protozoa and gastrointestinal nematodes suggest that long-term immunity induced by vaccination is a realistic goal for control of parasitic infections.

Introduction

The ability to induce immunologic memory is the key to the development of successful vaccines, which have been pivotal in controlling infectious diseases that have plagued humans and animals for centuries. The most famous example is the eradication of smallpox in the 20th century, which inspired a global campaign to control several other important infectious diseases (e.g., measles, polio) by large-scale vaccination programs.[1] The success of those campaigns begs the question why vaccines for all infectious diseases have not been developed; it is particularly notable that there are no vaccines for any of the parasitic diseases that cause tremendous morbidity and mortality in large parts of the world. In short, the answer is that we do not have a good understanding of how immunologic memory is established or maintained, a deficit that is particularly evident when it comes to T-cell memory. Without a framework for understanding memory, the development of vaccines continues to rely on a trial and error approach. This has clearly not been successful for developing parasite vaccines.

Fortunately, over the last decade a renewed interest in understanding the cellular and molecular basis of memory has generated a substantial amount of new information about immunologic memory. New tools, including the ability to monitor specific T cells with tetramers, adoptive transfer of TCR transgenic cells and intravital imaging techniques, have helped define the in vivo life history and biology of T cells during infection or following immunization. Based upon the results of these studies several models have been proposed that attempt to explain memory cell development and maintenance.[2-6] In this chapter we will explore some of the advances in our understanding of immunologic memory in parasitic infections, specifically

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focusing on recent studies that tell us about the memory T-cell subsets that mediate immunity to parasitic infections.

The future of vaccines for parasites looked particularly bright in the 1980's and 90's. The molecular revolution led to the identification and large-scale production of parasite antigens that previously were impossible to obtain in any reasonable quantity. At that time, the strategy for vaccine design was quite simple: identify the dominant antigens recognized by the immune system, clone those antigens and use them to induce a protective immune response. However, this approach turned out to be less successful than anticipated. The most notable failure was in the field of malaria vaccine development. It was known from pioneering studies in the 1960s that irradiated sporozoites could provide protection against malaria and it was later shown that this immunity was directed against the major surface antigen of the sporozoites, known as the circumsporozoite protein.[7,8] Cloning the circumsporozoite protein from malaria was prematurely heralded as the first step in what was thought to be the rapid development of a malaria vaccine. Unfortunately, there is still no malaria vaccine. However, while the malaria vaccine was not immediately forthcoming, the ability to clone this parasite molecule and subsequently many other malaria proteins, was a key advance on the pathway to a vaccine.

The other important advance that occurred in the 1980's was the discovery that $CD4^+$ T cells could be separated into subsets, termed Th1 and Th2 and that these subsets performed distinct immunologic functions.[9] Studies of the immune responses to parasites played a key role in elucidating the factors that control T-cell development. Notably, the differential development of Th1 and Th2 cells following infections with the protozoan parasite *Leishmania major* demonstrated the key role these subsets played in the development of immunity.[10-12] As it became clear that cytokines associated with the innate immune response could preferentially direct the development of T-cell subsets, there was a greater focus on understanding the role of adjuvants as inducers of innate cells that could influence the response to a vaccine. This led to many important advances in our understanding of how microbial products—what Janeway once referred to as the "immunologists dirty little secret,"[13]—influence the immune response. This led to the notion that if the appropriate immune response was stimulated by modulating the cytokine milieu, then a vaccine would be successful. In the case of leishmaniasis, it was found that inclusion of IL-12 in a vaccine could successful induce a protective immune response.[14] However, the duration of the immunity induced was limited and thus, simply inducing the appropriate response did not lead to long-term immunity.[15,16] While understanding the different subsets of T cells will be important in vaccine development, these results demonstrate that there is more to immunologic memory than simply inducing an appropriate effector response.

The failure to develop vaccines for several of the most important pathogens causing disease today, in spite of our ability to clone and produce critical antigens and our increased understanding of effector T-cell subsets, has led to a re-evaluation of what is required for immunologic memory. The simple notion that memory T cells represent the few cells left after an effector response has dissipated has been replaced by more complicated models of memory T-cell development. It is now apparent that the memory T-cell pool is heterogeneous and contains several types of memory T-cell subsets. One subset has the characteristics of effector cells, while another has been proposed to act as a reservoir of antigen-specific T cells that can expand upon rechallenge, differentiate into effector T cells and replenish the effector cell population (see Fig. 1). These latter cells, termed "T central memory cells" by Lanzavecchia,[2] express the adhesion molecule CD62L (L-selectin) and the chemokine receptor CCR7, which target the cells to the lymph nodes. The former subset, termed "T effector memory cells", develops from effector T cells, produces effector cytokines (such as IFN-γ or IL-4) and migrates through the tissues. Thus, effector memory T cells have the ability to immediately respond to a challenge infection, while central memory T cells proliferate in the lymph nodes and can replenish the T effector pool. Memory T cells would be expected to express the IL-7R, since IL-7 provides survival signals to T cells. On the other hand, effector cells would be expected to be short-lived. Understanding how these memory T cells develop and are maintained will be critical in the development of vaccines for parasitic infections.

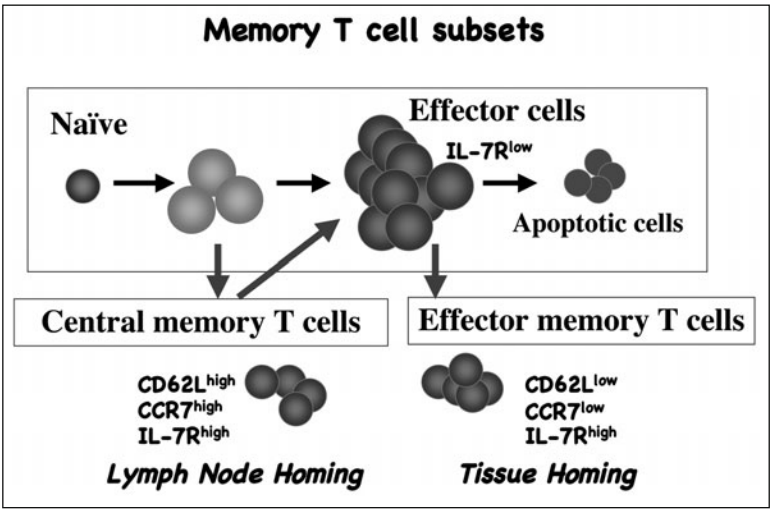

Figure 1. Heterogeneity in memory T cells. Naïve T cells circulate through the secondary lymphoid organs and are able to enter lymph nodes because they express CD62L and CCR7 on their surface. Upon contact with the appropriate antigen-presenting dendritic cells, the T cells proliferate and have the potential to differentiate into effector T cells that produce effector molecules (such as IFN-γ in the case of $CD8^+$ and $CD4^+$ Th1 cells or IL-4 in the case of $CD4^+$ Th2 cells). These effector cells lose expression of CD62L and CCR7 and gain expression of other adhesion molecules that target them to the tissues. While most of these effector T cells will undergo apoptosis, some will remain as "effector memory cells." Following activation of naïve T cells, some will cease proliferating before become effector cells. These cells express CD62L and CCR7 and thus, maintain the ability to circulate through the lymph nodes and are termed "central memory cells." This pool of antigen-reactive T cells acts as a reservoir of expanded antigen-reactive T cells, which upon reencounter with antigen can proliferate and become effector T cells.

What Are the Challenges for the Development of Parasite Vaccines?

Since there are vaccines for many viral and bacterial infections, one might conclude that there are unique hurdles to vaccine development for parasites. While not necessarily unique, there are hurdles that are more common within this group of organisms as compared to viruses or bacteria. Parasites frequently have complicated life cycles and immunity may need to operate at more than one stage of infection. They also can occupy niches within the host where our understanding of the immune responses is limited (e.g., nemotodes living in the gastrointestinal tract). In some cases, it remains unclear what the appropriate immune response is to control the parasite. Moreover, while antigens from various parasites stages have been identified, whether they function as dominant antigens is often unknown. Indeed, in the case of $CD4^+$ T cells it is not clear that there are overwhelmingly dominant antigens and it may be that protection is obtained simply due to reaching a threshold of antigen-specific T cells recognizing a diverse repertoire of antigens. In addition to the lack of dominant parasite antigens (or at least few defined dominant antigens), several parasitic infections (e.g., malaria, African trypanosomiasis) exhibit antigenic variation, which further complicates vaccine development. Another common characteristic of parasites is that they often induce chronic infections and associated with this chronicity is the development of many immunoregulatory mechanisms. How these modulate the immune response when immunized individuals are challenged has not been fully evaluated.

An important issue that may make vaccine development for parasites difficult is that control of these organisms most often requires T cells rather than antibodies. The vaccines in use today

mostly rely on generating high titered antibody responses, which is thought to be more easily achieved than protective T-cell responses that are maintained long-term.[1] In fact, in some cases it is still an open question as to whether immunity to parasites can be achieved with vaccination, or whether immunity may require the persistence of the pathogen (or the antigen).[17] The question of whether antigen is required for maintenance of memory T cells has been debated for years despite the fact that studies of both natural and experimental viral and bacterial infections indicate that it is unlikely that persistent antigen is required for maintaining memory T cells. However, it is critical to distinguish between maintenance of memory T cells and immunity to reinfection since these may not always correlate.[18] Whether resistance to reinfection requires the constant generation of effector T cells or can depend upon memory cells that may need more time to become effector cells probably depends upon the pathogen. For example, it has been suggested that immunity to malaria sporozoites may require the constant presence of effector cells since sporozoites are present for such a short time in the host.[19]

In natural parasitic infections the number of organisms associated with an infection may vary dramatically, which may also be important in the outcome of infection. How the challenge dose influences the ability to recall an immune response is not well defined. If a memory response requires additional expansion of the T cells, possibly by central memory T cells differentiating into effector cells, then low doses may fail to trigger this response effectively.[20] Similarly, how an immune individual responds to several low dose challenges is poorly understood and this type of exposure is often common with parasitic infections. One example where this may be an issue is in malaria endemic regions, where there is continuous exposure to mosquitoes transmitting low doses of sporozoites. Likewise, helminth infections in endemic regions are more likely to be initiated by trickle infections, which are known to stimulate different responses than higher, single dose infections.[21] Finally, protozoa replicate much slower than viruses or bacteria and in the case of helminths fail to replicate at all within the mammalian host, which is likely to have implications on how well an immune response is recalled upon challenge. Thus, taken together, the characteristics of parasitic infections present substantial hurdles for the development of effective vaccines.

Th1 Immunity: Balancing Resistance and Persistence in Parasitic Infections

While it is known that both $CD4^+$ Th1 cells and $CD8^+$ T cells contribute to the control of intracellular pathogens, how these T cells are maintained so that they can mediate long-term immunity to reinfection is poorly understood. Since many intracellular protozoan parasites, such as *Leishmania*, *Toxoplasma* and *Trypanosoma cruzi*, are associated with the long-term persistence of low numbers of parasites, it has been difficult to determine whether true memory T cells develop in such infections. Indeed, the argument has been made that immunity cannot be maintained in the absence of a low level of persisting organisms which in turn maintain a pool of activated effector T cells.[22,23] Since the strong resistance to reinfection associated with these infections may simply be due to the continuous generation of effector T cells, the protective immunity elicited by live infection may be difficult to replicate in the form of a vaccine. Fortunately, recent studies that take advantage of genetically modified parasites and chemotherapeutics suggest that similar to viral and bacterial infection, memory T cells can be generated and maintained independent of the persisting parasites.

Studies with parasitic protozoa have begun to incorporate the findings from viral- and bacterial-induced models of memory T-cell development with the phenotype of T cells responding to and mediating protection to parasitic infections. One infection which has been extensively studied as a model for the development of T-cell subsets is leishmaniasis. Leishmaniasis refers to a broad spectrum of disease states that can be induced upon infection with the more than 30 species belonging to the genus *Leishmania*. Seminal studies carried out in models of cutaneous leishmaniasis in which different strains of mice were infected with *L. major* were instrumental in defining the factors that promote $CD4^+$ Th1 and Th2 responses.[24] Specifically, infection with *L. major* leads to a Th1 response and resolution of infection in several strains of mice (e.g., C57BL/6),

while in other strains (BALB/c), a Th2 response is induced and progressive disease occurs. The most important influence on the outcome of infection with *L. major* is the presence of specific cytokines. For example, resolution of infection during a Th1 response requires the production of IFN-γ, which promotes the macrophage activation required for parasite killing. Moreover, IFN-γ production is dependent on IL-12 production as *L. major* infected IL-12 deficient C57BL/6 mice develop a Th2 response and are unable to control their disease.[25,26] In contrast, in the absence of IL-10, BALB/c mice are able to resolve a normally fatal infection and C57BL/6 mice heal more rapidly,[22,27] suggesting that IL-10 normally functions to inhibit immunity.

The quest to develop an effective leishmaniasis vaccine has been ongoing for many years, but unfortunately, all trials against human disease have been unsuccessful thus far. Nevertheless, resolution of a primary infection with *L. major* leads to life-long immunity. This is the basis of leishmaniazation, the only successful strategy that has been used to induce resistance to cutaneous leishmaniasis. This procedure involves the intentional inoculation of people with live parasites at an unobtrusive site of the body. Following resolution of the infection, individuals are resistant to reinfection. While for the most part discontinued due to the potential for serious complications, the efficacy of this approach directly demonstrates that strong immunity can be generated by infection. However, whether the low numbers of parasites that persist following infection are necessary to maintain immunity is an open question. Many experimental leishmaniasis vaccines in mice have been described, but only a few of them have examined the ability to induce long-term immunologic memory.[28] For example, IL-12 was able to promote Th1 cell development and subsequent immunity to challenge infection when used as an adjuvant in a *L. major* vaccine,[14] but subsequent studies showed that the immunity induced was short-lived.[15] These results and the failure of other experimental leishmaniasis vaccines suggest that a traditional leishmaniasis vaccine may be difficult to develop. Indeed, C57BL/6 IL-10 deficient mice are able to completely clear parasites following *L. major* infection and in contrast to wild-type mice, lose their immunity to reinfection.[22] This observation suggests that persistent parasites may be absolutely required for the maintenance of immunity and that delivery of a dead protozoal vaccine would not provide long-term immunologic memory.

Infecting mice with a genetically modified strain of *Leishmania*, termed *dhfr-ts*, was used to address this issue. These parasites lack the gene for dihydrofolate reductase-thymidilate synthetase[29] and hence are unable to synthesize thymidine. These parasites do not replicate in vivo and induce no apparent signs of disease in mice.[30] Importantly, the parasites are completely cleared from mice by 8 to 10 weeks post-infection. Infecting mice with wild-type (WT) *L. major* or *dhfr-ts* parasites allowed for a comparison of $CD4^+$ T-cell responses during chronic and nonchronic infections. Mice infected with WT parasites contained two types of $CD4^+$ T cells, distinguished by their expression of the LN-homing molecule CD62L.[31] One subset of cells, which expressed CD62L and homed to the lymph nodes, had the characteristics of central memory T cells. These T cells did not produce IFN-γ and moreover had the capacity to differentiate into either Th1 or Th2 cells depending upon their environment.[32] Recent studies indicate that central memory T cells develop early after infection with *L. major* (Colpitts and Scott, manuscript in preparation). A second population of cells had the characteristics of effector $CD4^+$ T cells. While both of these populations could transfer immunity to naïve recipients, the $CD62L^{lo}$ effector T cells could mediate resistance faster than the $CD62L^{hi}$ central memory T cells. In contrast to infection of mice with WT parasites, following infection with *dhfr-ts*, there was no evidence that an effector T-cell population (or an effector memory T-cell population) was maintained. However, $CD62L^{hi}$ central memory cells were maintained and were able to provide protection in *dhfr-ts*- infected mice or following adoptive transfer.[31] These results indicate that while parasites may be required for the maintenance of the effector pool, functional memory cells can be maintained in the absence of persistent parasites.

Studies with another intracellular protozoan, *Trypanosoma cruzi*, has shed light on the memory $CD8^+$ T-cell subsets that are associated with a chronic parasitic infection. Resistance to *T. cruzi* has long been known to be dependent on $CD8^+$ T cells.[33,34] However, it was unknown whether these $CD8^+$ T cells could be maintained in the absence of *T. cruzi* and whether they would have

the phenotypic and functional characteristics of memory $CD8^+$ T cells described with other types of pathogens. In order to address this issue, Tarleton and colleagues drug-cured infected mice and used a *T. cruzi*-specific tetramer to examine the pool of antigen-specific $CD8^+$ T cells that are present in chronically infected mice versus those that have achieved sterile cure.[35] Several important observations were made in these studies. First of all, following sterile cure of *T. cruzi*, a memory T-cell population was readily apparent that could mediate resistance to reinfection. Second, these cells had the characteristics of central memory T cells ($CD62L^{hi}$ $IL7R^{hi}$). This observation was in contrast to the majority of antigen-specific cells present in the chronically infected mice that exhibited an effector phenotype ($CD62L^{lo}$ $IL7R^{lo}$). As the effector cells present in chronically infected mice maintained their ability to produce the effector cytokine IFN-γ, these results also demonstrate that, in contrast to some chronic viral infections, chronicity of the pathogen is not always associated with the loss of functional T cells.[36,37] It is important to note that the central memory T-cell pool is not completely absent in chronically infected mice as a small population of $IL7R^{hi}$ cells were identified in the animals with persistent parasites. Some of these were also $CD62L^{hi}$ and had the capacity to produce IFN-γ following restimulation.[38] Thus, this observation suggests that, similar to *L. major* infections, central memory $CD8^+$ T cells may develop early after infection.

Taken together, the results of recent studies with *L. major* and *T. cruzi* indicate that both $CD4^+$ and $CD8^+$ central memory T cells can develop in the presence of parasites, suggesting that they may be part of the normal primary immune response to infection. In the case of *T. cruzi*, the $CD8^+$ central memory T cells were very effective at inducing resistance. However, in leishmaniasis central memory $CD4^+$ T cells were less effective at providing immunity to rechallenge. Whether this reflects differences in $CD4^+$ and $CD8^+$ T cells or different requirements for control of *Leishmania* and *T. cruzi* is unknown. In both cases, however, central memory T cells could be maintained without persistent parasites, suggesting that it should be possible to develop a vaccine for these infections.

Th2 Immunity: Longer Lasting Than Th1 Memory?

Gastrointestinal nematodes infect more individuals than any other group of parasites and no vaccines to control these parasites are currently available. Two well-studied gastrointestinal nematodes are *Trichuris muris* and *Heligmosomoides polygyrus*, both of which are controlled by Th2 responses. These parasites differ in their ability to persist long-term and are excellent models for studying protective memory responses induced under Th2 conditions. *T. muris* can be completely cleared from the gastrointestinal tract of resistant strains of mice, thus achieving sterile cure and subsequent immunity to reinfection.[39] Similar to the findings with *L. major* and *T. cruzi*, studies indicated that following clearance of *T. muris* infection a $CD4^+$ central memory T-cell population was maintained. However, unlike the findings with parasitic protozoa, the clearance of *T. muris* did not lead to the loss of effector T cells.[40] Moreover, by using IL-4 reporter mice, it was shown that some of the $CD62L^{hi}$ central memory T cells were already predisposed to Th2 cytokine production. Thus, these experimental infections with *T. muris* indicate that Th2 memory T cells may differ in fundamental ways from the memory cells induced during a Th1 response. First, Th2 effector memory T cells were maintained in the absence of the worms, while in leishmaniasis Th1 effector memory T cells were not maintained. Secondly, some of these IL-4 producing cells were able to express CD62L, which would allow them to home to the lymph nodes. While it is not yet clear whether these differences relate to the types of infection or may reflect a universal difference between the generation of memory during a Th1 versus a Th2 response, the data clearly indicate that the maintenance of an effector memory $CD4^+$ T-cell population does not always require persistent parasites.

Primary infection with *H. polygyrus* results in chronic infection, but the worms can be cleared following treatment with helminth-specific chemotherapeutics. These treated mice are subsequently resistant to a secondary infection.[41] Again, studies using an IL-4 reporter mouse were helpful in demonstrating that following clearance of the parasites memory Th2 cells were maintained.[42]

These findings, in conjunction with those described above for *T. muris*, further support the idea that Th2 memory may be fundamentally different from Th1 memory.

The ability to better resist a secondary challenge will depend not only on the presence of memory T cells, but also on the ability of those cells to induce effective effector functions. While it is well established that T cells are required for resistance to gastrointestinal nematodes, the effector mechanisms that mediate protection have been poorly understood. Recent elegant experiments indicate that alternatively activated macrophages (AAMϕ), which are characterized by the expression of the IL-4 receptor and the mannose receptor (CD206) and the production of arginase, are recruited to the host-parasite interface following secondary challenge with *H. polygyrus*.[43,44] Since the depletion of AAMϕ using clodronate-loaded liposomes induced a significant increase in parasites in the lumen and recovered larvae compared to control treated mice, this study establishes AAMϕ as critical for immunity. Understanding how memory influences the activation of effector mechanisms and what those effector mechanisms are, will be critical in developing vaccines for gastrointestinal nematodes.

Memory T Cells in Malaria

Malaria, along with tuberculosis and HIV/AIDs, is one of the three most important infectious diseases in the world today. While control of the mosquito vector and the development of chemotherapy have had a major impact on the disease, malaria is still responsible for the deaths of 1 to 3 million people per year. The development of a vaccine for malaria has been the goal of scientists for more than 40 years, but to date, they remain unsuccessful. Malaria exhibits many of the hurdles to vaccine development described above: it has a complicated life cycle, individuals may require continued exposure to parasites to maintain resistance, the parasite undergoes antigenic variation and multiple antigens are involved in protection. In addition, there is a belief that protective immunity is difficult to acquire naturally and that it is short-lived. However, this may depend upon how immunity is defined, since it has recently been argued that if resistance to severe disease is an indication of immunity then it can in fact be maintained for a long time.[45]

Malaria's life cycle involves a pre-erthrocytic stage where mosquito injected sporozoites migrate to the liver and invade hepatocytes. Here they replicate and eventually are released to go on and infect erthrocytes. The organisms replicate within the erthryocytes and are eventually released to infect other red blood cells. Protective immune responses to malaria can be directed at either stage of infection and a role for $CD8^+$, $CD4^+$ and B-cells (specifically antibodies) in immunity to malaria has been well documented.[19,46]

Although the species of malaria that cause disease in humans (*Plasmodium falciparum, P. vivax, P. malariae, P. ovale*) do not infect mice, studies of murine malaria (e.g., *P. berghei, P. yoelli, P. chabaudi*) have provided insights into the immune responses to these organisms and how memory develops following infection. Using these murine models and following up with human infections, it was established that irradiated sporozoites induce protection to malaria challenge[7] and since this immunization induces sterile immunity, it is considered the gold standard for a vaccine. The protection can be mediated by $CD4^+$ and $CD8^+$ T cells, as indicated by depletion and adoptive transfer experiments, and is directed against the circumsporozoite protein found on the surface of sporozoites.[47]

In order to understand how T cells respond to malaria infection during this early stage of the infection, TCR transgenic mice recognizing an epitope found in the circumsporozoite protein were created.[48] Cells from these mice were transferred into naïve mice that were subsequently challenged with malaria and the expansion and contraction of the T cells was assessed. Since malaria has been thought to induce poor immunity, a logical question was whether the T-cell response to circumsporozoite protein following malaria infection might be quite different than normal T-cell responses to other pathogens. The results from the studies with these TCR transgenic T cells indicated that the response was similar to that observed with other infections or immunizations, suggesting that a lack of immunity is not likely to be due to the absence of memory T cells.[19,49] Rather, it has been suggested that due to the need for a rapid response, a large number of effector

T cells rather than memory T cells, may be required to mediate protection.[19,20,47] Interestingly, the most important factor determining whether the parasites were eliminated was the number of specific T cells, rather than whether they were effector or memory T cells.[47]

The immunity induced by irradiated sporozoites has no influence on the erythrocytic stages of the infection, as immunized mice challenged with infected red blood cell show no immunity. Antibodies and $CD4^+$ T cells mediate the protection directed against the erythrocytic stage.[50-52] Again, a useful tool to understand the $CD4^+$ T-cell response and the generation of immunity was the creation of a TCR transgenic mouse where the T cells recognized an epitope on the merozoite surface protein 1 (MSP-1). This antigen has been extensively studied in malaria and is associated with protection.[53] Studies using these TCR transgenic cells indicated that $CD4^+$ T cells can protect mice, but that the best protection is obtained when antibodies are present.[54] Thus, the major role for $CD4^+$ T cells in protection during the blood cell stage may be to act as helper T cells promoting antibody responses. Future studies will be required to better understand why malaria-specific $CD4^+$ T-cell responses are so low and what will be required to maintain these cells to promote immunity.[52]

It is difficult to study the development of memory T cells in an endemic population, but fortunately there are now new tools available that take advantage of the murine models of malaria. Future studies with these models, in combination with human studies when possible, will hopefully shed light on the factors that contribute to the generation of memory $CD4^+$ and $CD8^+$ T cells and link the presence of these cells with immunity to infection.

Conclusion

The ability to monitor T-cell expansion and contraction in vivo has provided a clearer picture of how memory T cells develop during parasitic infections. These studies indicate that similar to other infections, heterogeneous populations of memory T cells can be generated, some of which have the characteristics of central memory and effector memory T cells. However, depending upon the parasite, these memory T cells may or may not be effective at providing rapid resistance to rechallenge. The challenge now will be to determine how to generate those memory T cells that are required for protective immunity by vaccination. The important recent findings that immunological memory can be induced by parasitic infections—either in protozoal or gastrointestinal nematode infections—provides hope that this will be feasible.

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Chapter 12

Antigen Specific Memory T Cells and Their Putative Need for the Generation of Sustained Anti-Tumor Responses

Kory L. Alderson and William J. Murphy*

Abstract

Memory T-cell responses to cancer antigens may be an effective way to sustain long-term tumor-free survival. However, finding an effective vaccination strategy to induce memory T-cell responses toward tumor associated antigens in patients with existing disease has proven to be extremely difficult. Immune stimulation regimens have been combined with tumor vaccination in an attempt to boost the immune response resulting in better vaccine efficacy. In these instances immune stimulation alone has shown some promise as a primary tumor therapy, but has been less effective at eliciting long-term tumor immunity. Likewise, combining systemic adjuvant therapy with tumor antigen vaccination also demonstrated a lack of sustained anti-tumor immunity in cancer patients. In this review, we discuss whether the immune response generated during immune stimulation is appropriate for supporting memory T-cell generation or whether initial tumor regression and generation of sustained anti-tumor immunity have different immunological signaling requirements.

Introduction

Immunological memory has classically been defined by immunological response time. Upon rechallenge with the appropriate antigen, memory cells react more rapidly to destroy pathogens. Immunological memory has therefore been employed for the control of widespread disease through the application of many vaccination strategies. Various infectious diseases can be controlled or even eradicated by vaccination. However, some diseases such as cancer have remained difficult to vaccinate effectively against. The difficulty in generating an effective anti-tumor response through vaccination has primarily been attributed to the state of active disease. Therefore in this context, vaccination is being used a treatment regimen rather than for prevention.

Preventative vaccination to viruses such as those that cause polio and smallpox have almost completely eradicated these diseases that were once considered epidemic in the United States and in the case of smallpox it has been eradicated worldwide. A similar approach is being used for the prevention of Human Papilloma Virus (HPV) infection for the prevention of cervical cancer. Unlike smallpox and polio, cancer vaccines are for a disease that has slowly developed out of self tissues, in some cases well over 20 years.[1] This is problematic for two reasons; one is the weakly immunogenic nature of most nonviral tumor associated antigens and second is the lack of sufficient immune induction associated with cancer-induced immunosuppressive pathways.

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Cancer is a general term for hundreds of different diseases, which illustrate a major problem associated with its management.[1] Many different vaccination strategies have been explored for the treatment of various types of cancer, both in mouse models and clinical trials. Some have shown limited and potentially promising results limited by the types of cancers that respond.[1,2] Thus, more research is needed to continue the search for effective anti-tumor vaccines as a means to boost anti-tumor responses. Due to multiple mechanisms of immunological suppression, the generation of antigen specific immunity and memory to tumor associated antigens (TAAs) is difficult, especially considering that most tumor vaccines are administered once the disease has become a problem. Additionally, many immunotherapeutic regimens, even those that seemed promising initially, did not confer lasting immunity to the individual.[3] Therefore, it is possible that in the context of an existing tumor, two therapy regimens must be applied; one that targets the initial tumor and one that generates lasting antigen specific T-cell immunity to TAAs.

One example of an effective vaccine that has potential to impact cancer rates is the HPV vaccine.[4,5] Gaining FDA approval in 2006, this vaccine protects against the two most common strains of HPV (HPV16 and HPV18) which are responsible for 60-70% of all cases of cervical cancer in the United States annually.[6] Recent reports have suggested the HPV vaccine is ineffective against active HPV infections (www.cdc.gov/std/hpv). Although the evidence is not yet sufficient to make definitive claims, it is likely that vaccination of women who already have HPV will not result in similar protection against HPV induced cervical cancer.

One current dilemma regarding vaccination for cancer therapy is whether the vaccine should be designed to amplify an existing immune response to the tumor or create and boost a de novo immune response to cancer associated or self antigens. Futhermore, to what extent does an immune response exist to cancer associated antigens and at what stage of tumor development is this response generated? Thus far it has been difficult to find clinical vaccination strategies capable of conferring both efficient immunological memory and maintaining tumor free survival. For the purpose of this review, we will question memory T-cell responses and the potential advantage for their use in cancer immunotherapies. Reducing tumor burden and maintaining tumor free survival may have two different immunological requirements. Reducing tumor burden may require a rapidly developed effector cell with broad specificity. On the other hand, tumor free survival may require antigen specific memory to tumor or self antigens that keep the tumor at a manageable level. Here, we will cover evidence to suggest that T-cell memory is vital to long term survival without tumor related complications and call into question the ability to generate effective memory cells if potent systemic immune stimulation is applied for the treatment of cancer.

The Difficulties Facing Potent and Sustained Immune Responses to Cancer

Directing T-cell responses to cancer associated antigens is problematic. It is generally accepted that the weak antigenic property of cancer associated antigens is due to their arising from self tissues or being intracellular in expression. While lacking a sufficient antigen for immune recognition is true for the majority of tumors, it is not absolute. Tumors with strong viral antigen components, e.g., cervical cancer, Burkitt lymphoma and adult T-cell leukemia, among others escape immune control. This demonstrates the extent of immunosuppression that tumors employ to evade immune recognition.

Immune responses to cancer have been classically divided into innate and adaptive arms due to specificity. Natural killer (NK) cells are often considered as a natural defense mechanism against virally infected and neoplastic cells. Unlike T cells, NK cells are not restricted to MHC and are therefore effective against tumor cells which have downregulated MHC class I in order to escape immune recognition. The presence of NK cells is limited within tissues, which minimizes their effectiveness against solid tumors. While NK cells are an attractive candidate to help reduce metastases, their lack of antigen specificity translates to a lack of immunological memory and sustained responses.[7]

Adding to the complexity of tumor-immune cell interactions is that the tumor can utilize immunological suppression mechanisms both locally in the tumor microenvironment and/or systemically. Some tumors are proficient at inducing the production of Th2 type cytokines, which skew the local immune response away from a proinflammatory response toward a humoral immune response that may have little effect on tumor growth.[8] Tumor microenvironments have a significant population of inhibitory cell types e.g., regulatory T cells and myeloid suppressor cells.[9-11] Tumor associated macrophages (TAMs) can inhibit immune responses and are commonly found within tumors.[12] Additionally, tumor cells can upregulate suppressive or death ligands to reduce the function of migrating activated effector cells.[13-15] Systemically, tumors can promote myeloid suppressor cells that upon interaction with T cells in the lymph node or other peripheral lymphoid organs inhibit the generation of an anti-tumor response.[16] Thus, even if T-cell responses to the tumor occur, suppression may obviate protection.

Evidence for Immune Responses to Cancer in Man

Despite the many suppressive networks involved, there is an abundance of evidence both experimental and anecdotal to suggest the immune system is cognisant of developing tumor growth. However, the stage at which the tumor is "seen" by the immune system falls into question and may depend on the tumor type. Animal studies as well as isolation of human tumors have demonstrated the presence of tumor infiltrating lymphocytes (TILs) that may contain tumor specific T-cell populations displaying an activated phenotype within the tumor are usually tumor specific.[17] Additionally, there is a favorable association between the number of TILs found within a tumor and the prognosis of patients with many different cancers including but not limited to breast and colorectal.[18-22] However, in vitro studies have suggested that the effector capabilities of TILs are inexplicably lacking full potential.[23,24] Therefore, the enhancement of TIL function and numbers, potentially through the application of a vaccine or adjuvant therapy regimen, would be favorable to a patient.

Dudley et al have worked to utilize/augment the effector function of TILs by ex-vivo expansion with interleukin-2 (IL-2) for adoptive transfer into melanoma patients.[25] Although limited to patients with metastatic melanoma, the observations were promising in the beginning with substantial improvements to tumor burden. While initial results were promising, this therapy was unable to sustained long term (>2 years) tumor regression.[25] This demonstrated that while the presence of specific activated T cells may have been efficient at reducing the tumor burden to near undetectable limits, sufficient long term memory was not conferred and the tumor eventually escaped immune control, which may have been due to the induction of antigen loss variants.

Historically, there have been compelling examples of immune surveillance to human and mouse tumors. One such example is from two individual kidney transplant patients receiving a kidney from an organ donor that had been involved in a fatal car accident. Both recipients later developed metastatic melanoma tissue typed to the kidney donor. This recurrence of tumor was despite the donor having been in remission for well over ten years at the time of the accident.[1] The idea of immune surveillance has been supported using mouse models. It was demonstrated using a spontaneous prostate cancer model that while not all mice grew palpable tumors after carcinogen exposure, T-cell depletion resulted in tumor growth in nearly 100% of the mice that were previously tumor free. This study indicated that immunological suppression can maintain tumor burden at an undetectable limit.[26]

Immune Responses to Tumors in Mouse Models

Studies in immunodeficient and transgenic mouse models have demonstrated a direct role for immunological recognition in tumor destruction and in shaping the immunogenictiy of tumor cells during development. The observation that tumors in immundeficient mice occurred despite being house in specific pathogen free environments led to a hypothesis of tumor-immune cell interaction called "immunoediting".[27] Cancer immunoediting occurs in three stages; elimination, equilibrium and escape. During the elimination phase, cells of the innate and adaptive immune

system are capable of recognizing neoplastic cells and destroying them. Interferon-gamma (IFNγ) is an important effector cytokine for this process and spontaneous tumors rendered resistant to or sensitive to IFNγ signaling have been shown to be more or less responsive to immune destruction, respectively. Despite more recent data suggesting a cytostatic role for IFNγ induction by potent immune stimulation,[28] the role for IFNγ in tumor cell elimination was originally thought to be due to activation of effector T cells and to IFNγ induced upregulation of MHC I on tumor cells directly.[27] IFNγ upregulation of MHC I may be a key factor in tumor recognition and elimination as it may be instrumental in the ability of the normal cellular components becoming tumor associated antigens (TAAs).[27] During the second phase of cancer immunoediting, tumor cells which survived the elimination phase undergo consistent targeting and destruction by cells of the immune system. This can be beneficial or detrimental to tumor development. The equilibrium stage has the potential to last for a long period of time or to select for a less immunogenic variant of the tumor which can then enter into the third stage of tumor immunoediting, escape.[27] This demonstrates the potential dichotomy in the actions of immune cell recognition and targeting of tumor cells. It also demonstrates however, that continuous recognition may be needed not for eradication, but for control.

A compelling example of an acquired immune response to auto-antigens in the presence of tumor was recently demonstrated using a mouse model of prostate cancer. In spontaneous adenocarcinoma, Savage et al demonstrated that TILs from tumor bearing mice, unlike T cells isolated from their nontumor bearing counterparts, recognized a ubiquitously expressed self antigen, histone H4.[17] While the recognition of histone H4 did not result in complete tumor regression, it did result in a significant reduction in tumor size.[17] This report suggests that TILs are capable of recognizing normal cell components when associated with a tumor, which is contrary to what was previously believed. What is not addressed in this publication however is the role of histone H4 and why this specific intracellular protein was targeted by cells of the immune system. This leads to questions of when immune cells are capable of recognizing tumor cells and why the tumor is capable of escaping complete immune eradication.

Part of the aforementioned question was answered recently with the demonstration that carcinogen induced tumors are not only recognized by the T-cell repertoire of mice, but that immunological recognition is capable of maintaining a state of tumor "equilibrium".[26] It was observed that after carcinogen exposure, approximately 20% of the exposed mice never developed tumors. However, upon depletion of T cells (both $CD4^+$ and $CD8^+$) from the nontumor bearing cohort, sub-cutaneous tumors quickly became apparent. From these T-cell depletion studies it was concluded that immune controlled tumor equilibrium was responsible for the lack tumor progression in the original "tumor free" cohort. Furthermore, the authors demonstrated a role for immunoediting of tumor antigens by tumor transfer studies from tumor bearing mice into naïve recipients. Tumors which developed spontaneously as opposed to those that developed as the result of T-cell depletion had different levels if immunogenicity and therefore displayed different growth rates.[26]

Tumor stroma is another tissue to which an immune response can be generated to maintain a state of equilibrium.[29,30] Using antigen specific $CD8^+$ T-cell adoptive transfers, Zhang et al demonstrated tumor regression when tumor stroma cells were pulsed with tumor antigen such that the tumor antigen was cross presented to T-cell infiltrating the site.[29] Furthermore, the transferred $CD8^+$ T cells maintained a state of cancer equilibrium and consistently destroyed myeloid derived stromal cells.[30] This demonstrated that directing antigen specific responses toward the tumor stroma and not the tumor cells directly may be capable of maintaining tumor load to a minimum.

Taken together these publications have demonstrated the degree to which the immune system can play a role both in the emergence of tumors as well as the recognition of tumor associated antigens. However, the question still remains; how do the tumors bypass such immune control? How do they escape? Numerous studies have demonstrated that immune cell recognition can lead to tumor escape,[27] most likely through the emergence of an antigen loss variant. Additionally, Savage et al demonstrated that the immune system disregards the evolutionary pressures and can

generate immune responses to "self" determinants, including ubiquitously expressed "self" proteins at some as yet undetermined time after tumor initiation. However this still does not explain why tumor burden was not subsequently reduced after self peptide recognition or why severe autoimmunity was not observed.

While mouse studies are helpful in showing what can be done, they are limited in their mimicry of the human situation. Some of the primary flaws, which have the potential to be controlled, are that studies often utilize young mice which are inbred and results are rarely demonstrated in more than one strain. Additionally, the mice are housed under specific pathogen free conditions. The majority of the immune repertoire phenotype of a young (8-16wk old) mouse is of a naïve phenotype. Cancer predominantly affects elderly individuals and many studies have demonstrated that the aged immune system is significantly altered in comparison to the young immune system in its ability to generate an immune response.[31-33] So how does the aged immune system generate immunological recognition to the weak, self antigens necessary to induce tumor equilibrium? Importantly, are there means to augment this response and still have it sustained?

Is Immunological Memory Important for Tumor Regression or Tumor Equilibrium?

Thus far, we have discussed two potential problems that face effective vaccination to cancer antigens. First, cancer antigens are often weak in eliciting an immune response. Second, cancer antigens are generated over long periods of time, often without "danger" signals. The amount of time that cancer antigens are present may have an effect on the ability of immune cells to recognize and target them. One reason for this is peripheral tolerance. Peripheral tolerance is a mechanism by which T cells are tolerized to self antigen exposure in the periphery. One proposed mechanism for this is through self peptide expression by antigen presenting cells (APCs) in the periphery without appropriate cositmulation.[34] Another proposed mechanism of peripheral tolerance is the co-expression of inhibitory molecules such as B7-H1 alongside MHC-peptide on the surface of normal cells.[35] B7-H1 is a ligand for programmed death-1 (PD-1) which is inhibitory to T cells.[36] In addition to being a mechanism of peripheral and central[37]tolerance, B7-H1 has been shown to be upregulated by a wide array of tumors in vitro in the presence of the pro-inflammatory cytokine, IFNγ.[38] A lack of costimulation and resulting tolerance to cancer antigens may be the outcome of tumor cell presence for long periods of time without any sign of danger to the host. Danger signals which occur during infection with a foreign pathogen or as the results of a high level of cellular necrosis are required for appropriate immunological activation.[39] While many tumors can be associated with high levels of necrotic cell death, this often occurs at much later stages in cancer progression therefore the tumor antigen has become putative. In order to generate effective immunological memory against cancer antigens, vaccination strategies must overcome these mechanisms of T-cell suppression to self peptides that are associated with the tumor. However, it first needs to be determined whether immunological memory is effective for tumor regression and maintenance of tumor free survival.

The ex-vivo induction and transfer of IL-2 activated lymphocytes into a tumor bearing individuals can have profound effects on primary tumor regression, but has not demonstrated the ability to confer long lasting survival.[25] However, it is possible that optimal anti-tumor responses would be expected by combining highly activated T cells for initial tumor destruction followed by a regimen designed to generate immunological memory to maintain a state of tumor equilibrium. It has not been sufficiently addressed however whether tumor regression and tumor equilibrium require the same type of immune effector cell.

Using systemic adjuvant therapy to enhance the response generated to a tumor antigen vaccine has been the goal of many mouse models and clinical trials.[3] Toll like receptor (TLR) agonists are one such method of enhancing cancer vaccine efficacy. A TLR 9 agonist was used in melanoma patients in combination with a cancer vaccine strategy which resulted in a markedly higher expansion of melanin-A specific $CD8^+$ T cells in the peripheral blood of treated patients over that of the patients that received vaccine alone.[40] However, it was noted that the majority of antigen

specific T cells generated with TLR-9 agonist plus vaccine were of effector memory phenotype. Effector memory T cells do not persist for an extended period of time after infection and long term disease free status was not reported.[3] Systemic administration of interleukin-2 (IL-2) has been administered as part of many clinical cancer vaccine trials to support the antigen specific expansion of effector cells (reviewed in ref. 3). Most clinical trials utilizing IL-2 have been limited in the amount of cytokine that can be administered as IL-2 is associated with severe signs of toxicity.[41,42] However, with the exception of one report.[43] clinical trials using low dose IL-2 have not demonstrated a beneficial role to the addition of this cytokine to cancer vaccine regimens.[44-47] The administration of other systemic proinflammatory cytokines as well as the blockade of inhibitory cells and cell surface markers have also been used in conjunction with cancer vaccines in clinical trials to maximize vaccine efficacy.[3] Some have had modest results, but problems associated with toxicity are usually associated with effective enhancement of the immune response as well as difficulties in sustaining T-cell responses.

Lacking T-Cell Memory after Strong Immune Stimulation

There are two subtypes of memory T cells, effector memory ($CD44^{hi}$ $CD62L^{lo}$) and central memory ($CD44^{hi}$ $CD62L^{hi}$). The necessary signal(s) for a T-cell to become a specific subset is debated.[48,49] However, it is generally accepted that there is a different physiological role for each subset.[50] Central memory T cells (T_{CM}) are those typically regarded as immunological memory cells. They are described as the longer lived memory cell and are at their most basic definition characterized by the expression of adhesion molecules (e.g., L-selectin and CCR7) in addition to the adhesion and classic memory cell marker CD44. Both CD28 and CD127 are suggested to be important for the longevity of memory cells and central memory T cells can persist in the lymph nodes long after an antigen is cleared.[51,52] While the long term presence of $CD28^{null}$ T cells, both $CD4^+$ and $CD8^+$, have been described in humans, they have been linked with immune incompetence associated with normal aging or with chronic inflammation.[53] Upon secondary antigen exposure, central memory T cells rapidly produce cytokines and undergo a high level of cellular proliferation.[48,49] Conversely, effector memory T cells do not persist for a long time in mice and do not home to the lymph nodes. Mouse models have demonstrated that effector memory T cells persist rather for a relatively short period after antigen exposure, only lasting about 2-3 weeks.[50] Effector memory T cells are classified by their expression of the memory marker CD44 and their appreciable lack of other adhesion molecules, most notably L-selectin (CD62L). Effector memory cells also respond quickly to antigen re-exposure most likely with less rapidity and potency than central memory cells[49] (Fig. 1).

The powerful effector capabilities of memory $CD8^+$ T cells make them an exceptionally attractive candidate for cancer therapy. While some tumor models seem to be directly sensitive to the killing capabilities of $CD4^+$ T cells,[54] these cells are not generally regarded as potent effector cells in mouse tumor models. However, $CD4^+$ T cells are vital to the generation and possible the maintenance of effective $CD8^+$ T-cell-mediated immunity, particularly memory $CD8^+$ T cells, which may necessitate their presence in therapy approaches.[55-59]

To understand T-cell memory it is important to understand the critical collaboration between $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells are known for their functional role as helpers and have many times been shown to be needed during the primary immune response to prime $CD8^+$ T cells properly for sustaining immunological memory.[58,59] In various tumor models, our laboratory demonstrated that administration of systemic potent proinflammatory immunotherapy using an agonist mAb to CD40 in combination with high dose interleukin-2 (IL-2) resulted in increased $CD4^+$ T-cell death.[28] The increased $CD4^+$ T-cell death was correlated with an increased expression of the INFγ dependent PD-1 ligand, B7-H1.[60] This increased $CD4^+$ T-cell death correlated with a lack of continual memory to the tumor antigens when immunotherapy was administered directly following irradiated tumor vaccine.[28] The lack of sustained T-cell memory was despite primary anti-tumor immunity being previously observed with the same systemic immunotherapy regimen.[61] Effector cells generated during immunotherapy were predominantly effector memory

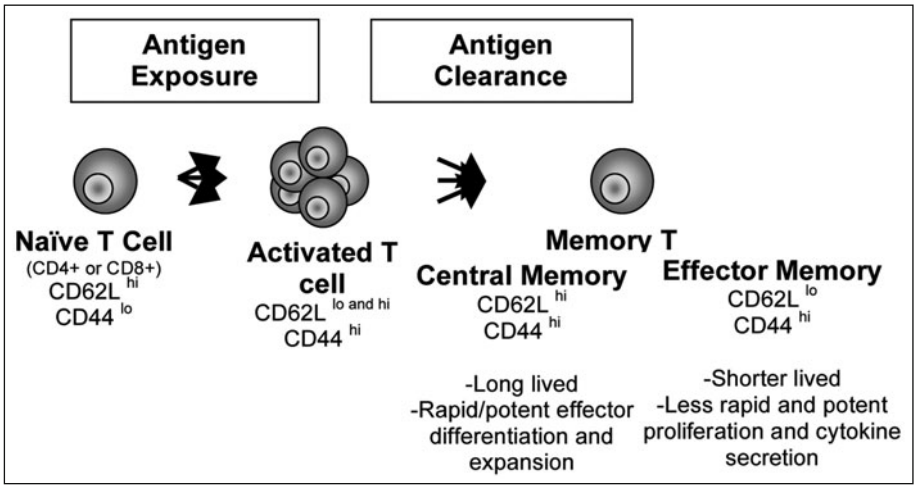

Figure 1. Generation of memory T cells. Two subtypes of memory T cells are generated following an immune response. Central memory ($CD62L^{hi}$ $CD44^{hi}$) T cells are longer lived and are activated more rapidly as determined by cytokine production and death ligand upregulation. Effector memory ($CD62L^{lo}$ $CD44^{hi}$) T cells are shorter lived and less rapid effector cells, but are maintained in the periphery for a extended period of time after infection.

phenotype, therefore it was possible that potent immotherapy only supported the initiation of immediately powerful effector cells and that immunological memory was not generated simultaneously. In this model of potent systemic immunotherapy, the observed level of $CD8^+$ T-cell expansion strongly argues against selective expansion of antigen specific cells which would have resulted in long term immunological memory. CD40 stimulation alone has been demonstrated to have potentially detrimental effects on viral antigen specific T cells, but was dependent on the virus.[62] Bartholdy et al demonstrated that the deletion of antigen specific $CD8^+$ T cells occurred with lymphocytic choriomeningitis virus (LCMV) infected mice resulted in delayed viral clearance. However, CD40 mAb administration to mice infected with vesicular stomatitis virus resulted in enhanced virus specific $CD8^+$ T-cell function correlating with rapid viral clearance.[62] These publications demonstrate a potential problem with combination immunotherapies, cytokine support of vaccination may be beneficial to the primary or metastatic tumor burden but detrimental to the generation of long lasting immunity. Why then, if potent immune stimulation can generate memory phenotype cells does this not correspond with long lived immunity (Fig. 2)? The answer may lie in that every augmentation of an immune response does not necessarily mean expansion of antigen specific cells. Verneris et al has reported a mechanism through which $CD8^+$ T cells can acquire MHC-unrestricted killing mechanisms after T-cell receptor (TCR) crosslinking and high dose IL-2 in vitro.[63] Cytotoxicity of these cells was displayed toward many different target cells in an NKG2D mediated fashion and was found to be dependent on the expression of the adaptor protein DAP-10. Importantly, DAP-10 was only upregulated in the presence of high dose and not low dose IL-2 which correlated with the high level of cytotoxicity.[63] This paper demonstrated a possible mechanism through which potent systemic immune stimulation could be eliciting a large population of effector T cells that are capable of primary tumor regression but not antigen specific in nature and therefore do not persist as long lasting memory cells as demonstrated in Figure 2.

Long term immunological studies in a mouse model of sepsis have shown that immediately following induction of sepsis, dendritic cells (DCs) were depleted from the lung and the spleen.[64] Furthermore, it was demonstrated that once the DC population returns, their function, as determined by IL-12 secretion, is severely depressed for over 6 weeks after sepsis onset.[64] This study

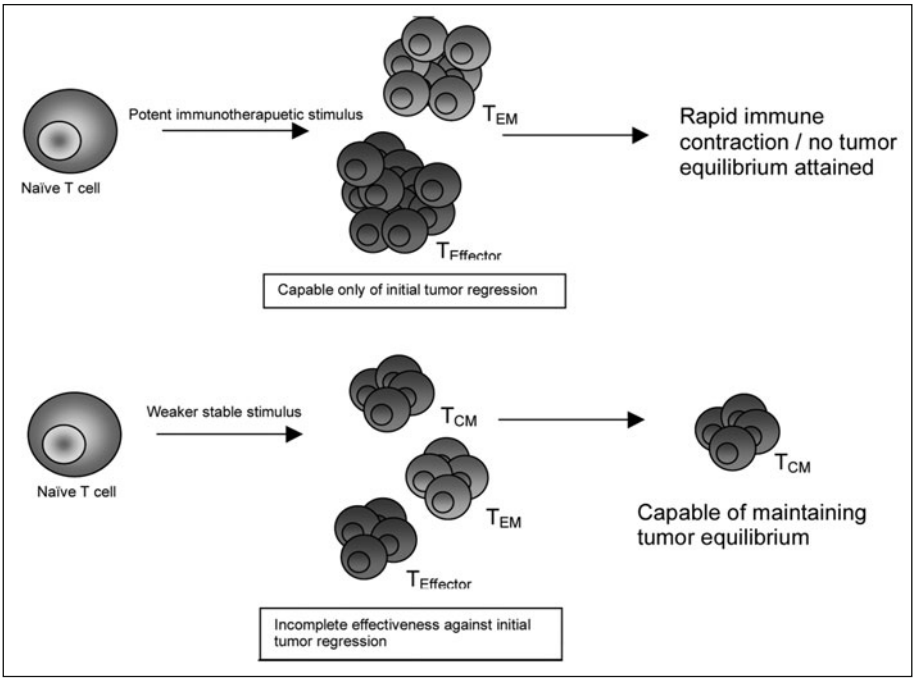

Figure 2. Divergence in memory retention depending on stimulus. This schematic demonstrates the hypothesis that potent immune stimulus initiates powerful effector memory (T_{EM}) and effector ($T_{Effector}$) T cells. This response is capable of inducing initial tumor regression, but long term memory is not attained to tumor antigens. Subsequently, weaker more stable stimulus elicits $T_{EM,}$ $T_{Effector}$ and central memory (T_{CM}) T cells which are capable of long lived antigen recognition and therefore tumor equilibrium.

may demonstrate an as yet unidentified problem associated with the administration of potent systemic immune stimulation for the treatment of cancer. A consequence of activation and expansion is contraction and loss without possible desensitization resulting in blunted responses and in cancer, relapse.

Conclusion

The examples shown herein may suggest a divergent role for immune cells in the reduction of immediate tumor burden and the long term maintenance of tumor free survival. While highly activated T cells seemingly eliminate a significant tumor burden, they may not be proficient at becoming memory cells and therefore lack the potential to support long-term tumor specific immunity. Conversely, memory T cells may not be sufficiently activated to eliminate a burgeoning tumor, but are proficient at keeping an already reduced tumor load at a minimum.

Utilization of vaccination effectively against tumors which have arisen from self antigens may require a more complete understanding of T-cell memory and its role during chronic state disease, such as cancer. Vaccination studies that utilize both antigen specific vaccine as well as the administration of strong immunomodulators, such as CTLA-4 blockade or cytokine, have shown some promising success in the clinic, but this may also be contingent on the immunogenecity of the cancer being treated.[3] Additionally, the length of response has yet to be determined. While the mechanism for these combination strategies has not been elucidated, combination strategies are aimed to result in independent initiation of effector and memory T cells however previous

studies have suggested that potent immune stimulus may destroy the generation of long term immunity to cancer antigens.[28]

Using vaccination to fight various cancers remains an area of strong interest to researchers as effective vaccination strategies can have profound effects on reducing the mortality associated with a specific disease. However, our knowledge is still limited regarding both the complexity of T-cell memory as well as the importance of immune control to self antigens. Recently, the use of mouse models has elucidated a direct role for immunological recognition in the destruction of tumor cells.[26] Additional studies have implicated strong immune stimulation as being beneficial to the primary response and detrimental to the secondary response.[28,60] This early beneficial effect may be through the generation of $CD8^+$ CTL that are relatively MHC-unrestriced in nature as previously demonstrated in vitro.[63] What remains to be addressed however is the long term consequence of large relatively antigen nonspecific expansion. Is the T-cell repertoire at a permanent disadvantage after this strong stimulus or can it regain its full potential? These questions demonstrate the need for more studies to elucidate if tumor therapy and tumor equilibrium can be generated simultaneously or if this needs to occur consecutively.

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Chapter 13

Memory T-Cell Responses and Survival in Human Cancer:
Remember to Stay Alive

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Abstract

Cancer is a major public health problem worldwide. Accumulating evidence suggests that tumor-host interactions may in part impact on tumor progression. However, the role of inflammation and adaptive immune reaction in cancer emergence, local and metastatic invasion and recurrence are still not clearly defined. Pro-inflammatory mediators are suspected to favor tumor growth and angiogenesis and naturally generated T cells with antigenic specificity to tumor associated antigens were usually in a state of anergy. Nevertheless, experiments in mouse and human showed a significant association between high density of tumor infiltrating T cells and improved cancer prognosis. Recently, the global analysis of colorectal cancer microenvironment demonstrated that a strong and coordinated Th1 adaptive immune response within primary tumors dramatically reduced the risks of relapse events. Interestingly the absence of early signs of metastatic invasion (lymphovascular emboli) correlated with a significant increase of the density of memory T cells in situ. This chapter presents the arguments supporting the existence of immunosurveillance mechanisms in human cancer. We will discuss the potent role of memory T cells in cancer immunity as well as the opportunities of therapeutic strategies uncovered by this new area of investigation.

Introduction

Many developments have occurred in prevention and treatment of cancer, but death from this disease is still common. Of the 58 million people who died worldwide in 2005, 7.6 million died of cancer. Based on projections, cancer deaths will continue to rise with an estimated 9 million people dying from cancer in 2015 and 11.4 million dying in 2030 (http://www.who.int/cancer/en/). Regardless of a great biological heterogeneity among malignancies, six major check points allowing tumors to adapt to their environment can basically describe cancer progression: (i) growth factor and (ii) proliferation-inhibiting signals autonomy, (iii) apoptosis escape, (iv) unlimited replication potential and—for carcinoma—(v) angiogenesis and (vi) primary tumor expansion and metastatic invasion.[1] Despite extensive characterization of the intrinsic[2] and environmental[1] underlying mechanisms, markers of the oncogenic process remain poorly predictive of patient survival and fail to prove their reliability in clinical use. Thus cancer prognosis is still estimated by yet imprecise classical anatomopathological parameters. For instance, the accuracy of current tumor-node-metastasis UICC-TNM staging[3] in colorectal cancer has remained largely unchanged

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since 1932 Dukes' original classification.[4] This lack of elements for relapse prediction improvement led to the investigation of the impact of nontumoral parameters on patient survival.

For immunologists, an hallmark of tumorogenesis is immune escape.[5] Because the immune system is in constant interaction with tumors, the ability to circumvent and adapt to immunosurveillance mechanisms[6,7] dramatically improves local and metastatic cancer progression.[8] Paradoxically, the immune system itself can participate in immune escape. Under the pressure of immune reaction a darwinian selection of variant tumor cells that are resistant to immune-surveillance mechanisms can occur. This is the model of immunoediting or immune shaping of tumors.[5,9] Inflammation is now commonly considered as a tumor-promoting factor.[10,11] Cancer cells can take advantage of the release into their environment of pro-inflammatory mediators such as TNFα, IL-1, IL-8 and IL-6 to increase their own growth and metastatic invasion and induce angiogenesis.[12-15] Thus, innate immune cells through inflammation-dependant mechanisms like tumor associated macrophages (TAMs) favor tumor progression.[16,17] Furthermore, inflammatory conditions can alter local immune responses. Indeed, intratumoral Type 2 macrophages (M2) were shown to produce high amounts of immunosuppressive cytokines IL-10 and TGFβ but low levels of Th1 cytokine IL-12.[18]

By contrast, experiments in mice revealed that immune responses mediated by IFNγ[19,20] and cytotoxic mediators such as perforine[21,22] secreted by lymphocytes[23,24] are involved in cancer immunosurveillance of solid tumors and lymphoma.[25] Local release of IFNγ can induces antiproliferative,[26] proapototic[27] and angiostatic[28,29] mechanisms leading to tumor cell death. Subsequent tumor antigen uptake, processing and presentation by APC to T cells can lead to antitumoral Th1 adaptive immune response induced and supported by IFNγ.[30,31] Consistently, in human cancer, infiltrating cytotoxic T cells were associated with improved clinical outcome and survival in melanoma,[32] ovarian cancer[33,34] and colorectal cancer.[35-39] In this chapter we will discuss the role of memory T cells in human cancer and the opportunities of therapeutic strategies uncovered by this new area of investigation. Because memory T cells are the final actors of the immune reaction cascade, they could represent a critical marker of antitumoral activity that may help establish cancer patient prognosis. Furthermore, due to strong cytokine secretion and cytotoxicity memory T cells may directly be involved in the control of tumor progression and metastatic invasion.

Characteristics of Tumor Antigen-Specific T Cells

Molecular identification of specific tumor antigenic peptides that started 20 years ago was decisive for the acknowledgment of adaptive immunosuveillance. The first human tumor associated antigen (TAA) was discovered by the team of T. Boon in melanoma.[40] The existence of TAA-specific T cells is now confirmed in an increasing number of malignancies (more information available at http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm). TAA and can be classified in 2 major groups: unique antigens[41] and shared antigens.[42] Unique tumor antigens result from point mutations in genes that are expressed ubiquitously but that are restricted to an individual patient or to very few patients. On the other hand, shared antigens are present on many independent tumors. They can be further divided into four groups: shared tumor-specific antigens, differentiation antigens, overexpressed antigens and viral and bacterial antigens. Shared Tumor-specific antigens correspond to peptides encoded by "cancer-germline" genes, such as MAGE, which are silenced in normal tissues but expressed in many tumors.[40,43] Differentiation antigens are not tumor-specific because they are also expressed in the normal tissue of origin of the malignancy such as tyrosinase, which is expressed in normal melanocytes and in most melanomas.[44] Overexpressed antigens are expressed in a wide variety of normal tissues but overexpressed in tumors. One of the most studied antigen of this group is the proto-oncogene HER-2/neu which is overexpressed in many epithelial carcinoma (ovary, lung, breast, etc.).[45] Viral and bacterial TAA are encoded by a number of pathogen agents such as Epstein-Barr virus (EBV) and human papilloma virus (HPV) that are associated with human malignancies.[46]

The quality of adaptive immune responses is tightly dependant of the quality of the T-cell repertoire that can be mobilized. Because TAA expression is generally weak, a high frequency of

naïve T cells resulting in high avidity and diversity of effector T cells are needed to elicit efficient antitumoral responses. Fortunately, all the data collected until now tend to indicate that the TAA-specific T-cell repertoire is much diversified independently of the antigen. A great polyclonality was observed among anti-Melan-A CD8 T cells.[47-49] Frequencies in the order of 5.10^{-7} were observed for CD8 naive precursors specific of MAGE-3 epitopes restricted by HLA-A1[50] or HLA-A2.[51] For comparison, the frequency of CD8 naive precursors specific of LCMV gp33 epitope in mice is 5.10^{-6} approximately.[52] When assessing the frequencies CD8 T cells in peripheral blood that were responsive to melanoma tumors, frequencies of 10^{-4} to 10^{-2} were observed with the highest values for Melan-A-specific T cells.[53-60] Frequencies of tumor-specific T-cell are increased (10^{-2} to 10^{-1}) in tumor invaded lymph nodes[60,61] and can represent up to 60% of infiltrating T cells in metastases.[57]

T cells responses against TAA remain poorly described because they were generally assessed by methods that did not allow extensive phenotypic and functional analysis. Limit-dilution followed by cloning protocols are limited by the duration of in vitro cell culture. Ex vivo studies lead to a better characterization of T-cell populations of high frequency such as Melan-A-specific T cells that may not be representative of all tumor-specific T cells. In peripheral blood, CD8 T cells that were specific of Melan-A epitope restricted by HLA-A2 presented intermediate states of activation and differentiation compared to T cells in invaded lymph nodes that had a more differentiated phenotype.[62] The profile of effector functions of tumor antigen-specific CD8 T cells remains controversial. Melan-A-specific T-cell populations from peripheral blood are able to secrete IFNγ ex vivo but present variable cytotoxic capacity.[63,64] CD8 T cells sharing specificity to a tyrosinase epitope were found functionally anergic in one study[58] whereas they were cytotoxic in another.[65] At the contact of tumors, T cells seem to enter in a state of functional tolerance[66] with impaired perforin and IFNγ secretion. The isolation from a melanoma metastase of a suppressor CD4+CD25+ clone specific to LAGE-1 antigen suggested a role for regulatory T cells in the induction of cancer tolerance.[67] However, this potent state of tolerance seems reversible in vitro.[66,68]

In summary, the identification of tumor antigens and associated specific effector T cells represents a great step in the revelation of anti-tumoral immunity mechanisms and offers promising perspectives for cancer immunotherapy. However, due to the experimental difficulty related with very low frequency of targeted clones, the comprehension of potent functional abnormalities in tumor antigen-specific T-cell responsiveness in regard of the pathologic state (expression patterns of tumor antigen, tumor microenvironment, etc.) remains largely unexplored. More global analytic approaches may help to answer these complex questions concerning host-tumor interactions.

Global Analysis of the Immune Reaction in Colorectal Cancer: A Breakthrough for Patient Prognosis

Th1 Adaptive Immune Responses and Patient Survival

It has been proposed that limitations of an experimental design can be overcome by integrating data obtained from two or more distinct approaches.[69,70] As described in *C. Elegans* and *S. Cerevisiae*, 'phenoclusters' can provide information about both the involvement of markers in particular modules and the functional relationships that might exist between them.[71-73] It is becoming clear that a global correlation of cellular and molecular datasets applies to the understanding of complex systems, such as tumor microenvironment and how biological hypotheses can be formulated based on data integration. Our team performed global phenotypic (large-scale flow cytometry), gene expression (Low density array RT-PCR) and topographic (TMA in specific tumor regions) analyses of the cells present in the tumor microenvironment of colorectal cancer (CRC) patients.[74,75] The concordant functional patterns of biological markers we observed led us to propose a new comprehensive view of anti-tumoral activity of immune cells.[76] Identified expression profiles of markers with potent implication in CRC outcome were validated, with a strong statistical confidence, in large cohorts of patients according to clinical reports and follow-up data that were prospectively collected and updated for 20 years.

We used large-scale flow cytometry to analyze subpopulations of immune cells from 39 freshly resected colon carcinoma.[74] To refine the analysis, 410 combinations of surface markers were measured. T cells, B cells, natural killer cells, natural killer T cells and macrophages were analyzed. CD3+ T cells were the most prevalent tumor-infiltrating immune cells. Naive T cells (CD3+CCR7+) were rare in the tumors. By contrast, in the differentiation pathway from early memory T cells (CD45RO+CCR7−CD28+CD27+) to effector memory T cells (CD45RO+CCR7−CD28−CD27−), all subpopulations were detected. We performed immunohistochemical analysis of tissue microarrays prepared from 415 colorectal cancers.[74] Kaplan–Meier curves suggested longer overall survival and disease-free survival among patients with tumors containing a high density of CD45RO+ cells than among patients whose tumors had a low density of such cells ($P < 0.001$ by the log-rank test). Patients whose tumors had a high density of CD45RO+ cells had a median disease-free survival of 36.5 months and a median overall survival of 53.2 months, as compared with 11.1 months and 20.6 months, respectively, among patients with tumors that had a low density of CD45RO+ cells ($P < 0.001$ for all comparisons). The respective five-year overall and disease-free survival rates were 46.3 percent and 43.1 percent among patients with tumors containing a high density of CD45RO+ cells and 23.7 percent and 21.5 percent among patients with tumors containing a low density of CD45RO+ cells. Multivariate Cox analysis showed that the M stage ($P < 0.001$), the N stage ($P = 0.002$) and the T stage ($P = 0.004$) as well as the CD45RO+ status ($P = 0.02$) were independent prognostic factors for overall survival. Thus, strong densities of memory T cells were associated with improved patient survival.

To assess the functional orientation of intratumoral responses, we used quantitative real-time polymerase chain reaction to evaluate the expression levels of genes related to inflammation, Th1 and cytotoxic adaptive immunity and immunosuppression.[75] These genes showed variable expression patterns in the 75 tumors studied. Correlation matrix followed by unsupervised clustering revealed highly significant combinations of comodulated genes, isolating clusters referring to known biological functions. Strikingly, a sole cluster for Th1 adaptive immunity (T-bet, IRF-1, IFNγ, CD3ζ, CD8α, granulysin and granzyme B) correlated with protection against relapse ($P < 0.05$). We did immunohistochemical analyses for CD3ζ, CD8α, granzyme B and CD45RO of tissue microarrays prepared from three independent series of colorectal cancer.[75] For each adaptive immune marker, there was a statistically significant correlation between immune cell density and patient outcome. These data suggested that Th1 adaptive immunity could have a beneficial effect on clinical outcome.

Altogether, these first observations indicated that strong intratumoral cytotoxic Th1 adaptive responses, illustrated by the expression of related mediators as well as the state of memory differentiation of T cells, could take place at the primary tumor site. These findings were inconsistent with infiltration of the tumor by inactive, anergic T cells indicating that cancer tolerance within tumors is not a generality. At the contrary, the strong pronostic values of the immune parameters we identified imply an efficient antitumoral activity of the immune system in situ. However, because high densities of T cells were not associated with the stages of tumoral tissue invasion, control of local tumor progression may not be the major role of immune reaction in situ. Conversely, infiltrates of high memory T-cell density were significantly associated with tumors without lymph node involvement and distant metastases ($P < 0.001$).[74] This strongly suggests that diminished risks of relapse occurrence are rather associated with control of the metastatic invasion process by the immune system.

Effector-Memory T Cells and Early Metastatic Invasion

Antitumoral activity of adaptive immune responses and improved prognosis associated with T-cell infiltrates of high density have been described in human colorectal cancer.[35-39] Evidence is accumulating that colorectal cancer expresses tumor-associated antigens (e.g., K-ras mutations, p53, carcinoembryonic antigen, Ep-CAM and SART3) that can induce tumor-specific T-cell responses in patients.[38] Recently, systematic analysis of genetic alterations identified 751 somatic

mutations in human colorectal cancer and derived cell lines,[77] emphasizing a wide spectrum of putative peptides for T-cell recognition. All these experimental data provide strong arguments in favor of the immune-mediated control of colorectal cancer. We chose to investigate the potent associations between immune and histopathological parameters. In regard of our preliminary observations we focused on the possible involvement of the immune system in the control of metastatic spreading.

We assessed the early steps of the metastatic invasion processes at the primary tumor site, which include vascular emboli (VE), lymphatic invasion (LI) and perineural invasion (PI) (collectively referred to as "VELIPI").[74] VELIPI status of tumors was determined from the histopathological reports obtained at the time of resection in a representative cohort of 959 patients. A VELIPI-positive tumor had at least one of these pathological findings, whereas a VELIPI-negative tumor had none of the three findings. The VELIPI status had a significant pronostic value. Kaplan–Meier curves suggested longer overall survival and disease-free survival among patients with VELIPI-negative tumors than among patients with VELIPI-positive tumors ($P < 0.001$ by the log-rank test for both analyses). There were significant differences in the median duration of disease-free survival between patients with VELIPI-positive tumors and patients with VELIPI-negative tumors (3.3 months vs 26.9 months, $P < 0.001$). A similar pattern was found for overall survival. Furthermore, the presence of more than one sign of early metastatic invasion conferred a worse prognosis than the presence of a single sign.

The association between signs of early metastatic invasion and immune reaction was evaluated by integrative analyses of complementary approaches.[74] Immunohistochemical analysis of tissue microarrays showed that VELIPI-negative tumors contained high numbers immune infiltrates and in particular of CD45RO+ cells as compared with VELIPI-positive tumors ($P = 0.02$). Large scale phenotypic analysis confirmed that the absence of tumor emboli was associated with a significant increase of (i) the density of intratumoral CD3+, CD3+CD4+ and CD3+CD8+ T cells (by a factor of 2.6, 2.5 and 4.9, respectively; $P < 0.05$) (ii) the expression of the marker of T-cell activation (HLA-DR, CD98, CD80, CD86, CD134), differentiation (CD45RO, CD45RA, CD27, CD28, CCR7, CD127), migration (CD62L, CCR7, CD103, CD49d, CXCR3) and (iii) the proportion fully differentiated CD8+ T cells. Finally, gene expression profiles revealed that the expression levels of cytotoxicity mediators (CD8α, granzyme B, granulysin) and Th1 adaptive response mediators (T-BET, IRF-1, IFNγ) were significantly increased VELIPI-negative tumors from patients who had not relapsed, as compared with levels in VELIPI-positive tumors from patients who had relapsed ($P < 0.05$). In contrast, levels of the Th2 transcription factor GATA-3, inflammatory mediators (IL-8, VEGF, CEACAM-1, MMP7, COX-2, thrombospondin-1) and immunosuppressive molecules (TGFβ, IL-10 B7-H3, CD32b) were not differentially expressed among the group of patients.

Our work, for the first time, provides the evidence that Th1 adaptive immune response elicited at the primary tumor site of colorectal carcinoma can control cancer recurrence and are beneficial for patient survival. Concordant data from distinct approaches indirectly indicate that early metastatic dissemination is probably hold in check by tumor infiltrating memory T cells through their superior effector function as compared to conventional undifferentiated T cells. However, it cannot be excluded that intratumoral lymphocytes modify tumor stroma or tumor cells, or both, in such a way that they attenuate the metastatic capacity of tumor cells. Furthermore, we failed to identify the biological mechanisms associated with reduced T-cell infiltration within tumors. Pro-inflammatory and immunosuppressive molecules we analyzed were not associated with immune cell recruitment at the primary tumor site. Thus, differences in tumor immunogenecity among CRC tumors still need to be elucidated.

In Situ Coordination of Immune Reaction and Improved Prognostic Evaluation

A note of caution is useful when interpreting the highly significant correlation we found between the quality of the in situ immune reaction and the signs of tumor dissemination and

clinical outcome. This indicates a strong but indirect evidence of an immune-mediated control of colorectal cancer progression. In an attempt to better evaluate the impact of T-cell infiltrates on the dissemination of metastases from the primary tumor site, we extended tissue microarray analyses to the immediate surroundings of the primary tumor—the invasive margin (IM)—in addition to the center of the tumor (CT).[75] Patients without recurrence had higher immune cell densities (CD3, CD8, GZMB and CD45RO) within each tumor region, than did those from patients whose tumors had recurred. In each tumor region (CT and IM) and for each marker (CD3, CD8, GZMB and CD45RO), there was a statistically significant correlation between immune cell density and patient outcome for a large range of cutoff values. In particular, using the cutoff that yielded the minimum P value for disease-free survival, the densities of CD3+, CD8+, GZMB+ and CD45RO+ cells in each tumor region (CT and IM) allowed the stratification of patients into groups with different disease-free survival rates (P values ranging from 10^{-2} to 10^{-6}) and overall survival rates (P values ranging from 10^{-3} to 10^{-8}).

The combined analysis of tumor regions further improved the prediction of patient survival. For all markers, the combined analysis of CT plus IM regions increased the accuracy of prediction of disease-free and overall survival time for the different patient groups, as compared to single-region analysis. These results were confirmed in two other independent cohorts of patients. Univariate and multivariate analyses done in multiple parallel ways (correction factors, median cutoff, cross-validation methods, leave-one-out and bootstrap methods) led into similar conclusions. These results strongly support the presumed immune activity against metastatic dissemination from the primary tumor site.

Finally, we determined whether these immune criteria could discriminate patient outcome at each step of cancer progression. Patients were stratified according to the UICC-TNM classification.[3] A strong in situ immune reaction in both tumor regions (CT and IM) correlated with a favorable prognosis regardless of the local extent of the tumor and of invasion of regional lymph nodes (stages I, II and III). Conversely, a weak in situ immune reaction in both tumor regions correlated with a poor prognosis even in patients with minimal tumor invasion (Stage I). Furthermore, patients with low densities of CD3+ cells and CD45RO+ memory T cells in both tumor regions had a very poor prognosis, similar to that of patients with concomitant distant metastasis (Stage IV). Multivariate Cox analysis showed that immune patterns remained the unique parameter significantly associated with prognosis, whereas T-stage, N-stage and differentiation of the tumor were not significant when adjusted with immune patterns. Thus, the amplitude of adaptive immune reaction within the primary tumor was a better predictor of survival than traditional clinical parameters.

A Long-Term Memory against Cancer?

Our results suggest that once human CRCs become clinically detectable, the adaptive immune response plays a role in preventing tumor recurrence. Despite immunoediting mechanisms, the beneficial effect of the adaptive immunity may persist throughout tumor progression (stages II and III). The absence of microscopic evidence of early metastatic invasiveness within lymphovascular vessels was strongly positively correlated with high densities of intratumoral effector-memory T cells. An appealing interpretation of these data is that even when a tumor has already reached an advanced clinical stage, efficient adaptive immune reaction can keep tumor emboli in check. Because cancers present the physiopathological characteristics of chronic and evolutive diseases, it is not surprising to observe differentiated memory T cell within tumors. Strong adaptive immune reactions resulting in massive recruitment and differentiation of T cells in situ can basically explain the strong prognostic value of CD45RO marker. However, it could be hypothesized that effector-memory T cells may not only illustrate global antitumoral immune responses but may be directly involved in the control of cancer progression. The cytotoxic and cytokinic capability of effector-memory T cells may provide them the relevant weapons to control tumor progression and metastatic invasion at the primary tumor site. In another hand, the observation of high intratumoral immune reaction in patients with advanced metastatic cancer could indicate that the

immune system is unable to efficiently prevent metastatic dissemination. Thus other antitumoral immune mechanisms may be implicated in reduced relapse occurrence.

Because the primary tumor is removed by surgery, the prognostic value associated with the host response in colorectal cancer may reflect a quality of systemic effectors for recognition and killing of circulating cancer cells in peripheral blood, peritoneal cavity, bone marrow, or lymph nodes. The effector-memory T-cell ability to "remember" previously encountered antigens leads to faster response on reexposure. Following a primary response to antigen, memory T cells disseminate and are maintained in the body for long periods.[78] As suggested in mice,[79] the trafficking properties and the long-lasting antitumor capacity of memory T cells could result in long-term immunity in human CRC.

It is suspected that metastatic invasion can lead to the dissemination of tumoral foci that can remain in an asymptomatic and nondetectable state of dormancy (i.e., not expanding in mass) for long periods of time before cancer reemergence.[80-82] Control of cancer dormancy involves various mechanisms like cellular dormancy (G0-G1 arrest), angiogenic dormancy and immunosurveillance.[83] Recently, Koebel and colleagues demonstrated that stable lesions of transformed immunogenic cells in mice were controlled by the host's adaptive immune system in a condition of 'equilibrium'.[84] In these experiments, loss of either immunocompetence or immunogenicity could lead to tumor outgrowth. Based on these data it could be hypothesized that human cancer relapse may arise either because of the loss of the protective antitumoral immunity and/or the 'awakening' of dormant tumors. This could explain why occult cancer can be transplanted from organ of a donor—apparently cured from cancer—to a recipient[85] who is at one and the same time naive to the transplanted tumor cell antigens and under immunosuppressant treatment.

In this context, our data suggest that depending on the strength and localization of antitumoral immune responses elicited in situ, distinct quantity (number of clones) and quality (memory differentiation state) of memory T cells could be generated among the patients. Before surgery, resident memory T cells may actively reduce the number of disseminating occult tumors. Following resection of primary and secondary tumors, circulating memory T cell may participate in the control disseminated distant occult tumors. During the equilibrium phase, both mechanisms may result in tipping the scale towards immunosurveillance mechanisms against occult tumor outgrowth, thus dramatically reducing relapse occurrence and favoring patient survival.

Perspectives for Cancer Research and Treatment Strategies

The strong prognostic value of immune parameters we uncovered has to be repeated in independent and larger cohorts of colorectal cancer patients and in those with other cancers. Similar observations are expected in melanoma but some data in breast cancer advocate against it.[86] However, if confirmed these results may have immediate impact on the establishment of cancer patient clinical prognosis. Indeed, the combination of immunological parameters together with classical clinical observations in routine may dramatically improve the prognosis of cancer patients. Redefined classifications according to patient immunologic profile may allow proposing more adapted treatments and therapy strategies. Indeed, this could help identify high-risk individual with modestly invading tumors who would benefit from aggressive adjuvant therapy as well as patients with advanced tumors but good immune criteria who may not necessarily need postoperative treatments.

At present, classical treatments against cancer (surgery, radiotherapy and chemotherapy) allow half the patient to durably survive their disease despite important adverse reactions. Cancer immunotherapy offers complementary strategies with minimal secondary effects.[87,88] However, direct tumor antigen vaccination strategies globally induce only marginal objective responses in clinical trials (3% according to RECIST criteria)[89] though in some cases evidence of tumor regression can be observed in up to 20% of enrolled patients.[50] This disappointing clinical inefficiency is probably inherent to stimulations of monospecific T-cell responses. Indeed, adoptive transfer of dendritic cells activated with tumor antigens are associated with 9.5% of global clinical responses,

a 3 time increase as compared to vaccination trials.[89] This suggests that costimulation molecules[90] and multiple epitope of a given antigen[91] presented to T cells by antigen presenting cells improve antitumoral immune activity. This is consistent with the observation that only strong and coordinated adaptive immune responses are associated with good prognostic in human CRC.[75] Thus, the key for immunotherapy success may reside in the induction of global antitumoral reaction in situ strong enough to circumvent the tumor immunosuppressive network. Furthermore, given the suspected role of memory T cells in the long-lived control of cancer reemergence, local tumor regression may not be the sole objective of immunotherapeutic strategies. The artificial induction of large and diversified populations of memory T cells may dramatically reduce the risks of cancer relapse following tumor removal or destruction.

The use of monoclonal antibody remains the most successful strategy of immunotherapy. Recent clinical studies (1998-2004) show that rituximab (anti-CD20) treatment alone induces 40-70% of partial responses and 4-37% of complete remission of lymphoma patients.[92] Besides, anti-VEGF treatments provide an alternative but complementary approach to reduce colorectal cancer progression[93] when combined with chemotherapy.[94] Angiogenesis in part mediated by VEGF expression seems to play a critical in primary tumor progression by promoting nutriment supply[95] and inducing vascular exit paths for migrating tumor cells.[96] A greater systemic mass of occult tumors may increase the risks of disruption of their dormancy state because of overwhelmed immunosurveillance mechanisms. Furtheremore, if the migrating tumor cells inherit the strong angiogenic properties of their resident counterparts the angiogenic dormancy mechanisms[97] may be dramatically impaired. Because angiogenic mechanisms may occur very early in CRC the onset of dysplastic transformation in the polyp[98] anti-VEGF treatments could be efficient even in early CRC stages. In regard of our results and hypotheses, combined treatment targeting both angiogenesis inhibition and memory T-cell generation could dramatically favor patient survival by (i) respectively reducing metastatic spreading and enhancing immune reaction within tumors and (ii) respectively enhancing occult tumor dormancy and long-term immunosurveillance during the equilibrium phase. Further studies are needed to investigate these crucial issues.

In the field of fundamental research, future comparative studies of tumors according to immune parameters may reveal distinct biological processes of tumor-host interactions such as tumor immunogenecity or immune escape and subsequent regulation of immune cell recruitment and activation in situ. Cataloging tumors with distinct immune sensitivity could facilitate the comprehension of emergence and cancer progression. This new area of investigation may help uncover individual and universal mechanisms of human antitumoral reactivity.

Conclusion

Continuous technologic advances in cancer models and immunodeficient mice, miniaturization of experimental tools and large scale analysis allowed uncovering several mechanisms of immunosurveillance and tumor escape. Integrative biology represents a valuable approach to assess the complexity of tumor-host interactions in situ. The beneficial impact of intratumoral Th1 adaptive immune response on patient outcome is a powerful argument in the demonstration of antitumoral immunity. In human colorectal cancer, immune parameters were even better predictors of patient outcome than classical anatomopathological parameters. At the primary tumor site, the positive correlation between the absence early metastatic events and high densities memory T cells was associated with prolonged patient survival. This provide a better comprehension of the role of memory T cells in local control of cancer progression and offers new appealing hypotheses in favor of potent mechanisms of long-lived cancer immunity. The combination of immunological parameters together with classical clinical observations in routine may dramatically improve the prognosis of cancer patients by redefining classifications according to their immunologic profiles. More adapted treatments and therapy strategies may ultimately be proposed to attempt to efficiently cure colorectal cancer.

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Chapter 14

Analysis of Vaccine-Induced T Cells in Humans with Cancer

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Abstract

Over the past several years, progress in the field of tumor immunology has lead to advances in active immunotherapy and vaccination as a means of eliciting tumor-specific immune responses to mediate tumor regression and clearance. Developing vaccines targeted against cancer became an important focus as a therapy following the success of viral vaccines in preventing infection and disease. In humans with cancer, similar to viral infections, the host immune system is capable of recognizing antigens expressed on tumor cells. This similarity allows the immunological framework of the viral vaccine to be adapted to the cancer setting in hopes of enhancing human T-cell reactivity against tumor.[1] It is generally believed that a requirement for tumor destruction to occur is the induction of sufficient levels of immune cells with high avidity for recognition of tumor antigens. Moreover, the cells must be targeted to the tumor site and be capable of infiltrating tumor stroma.[2] Several tumor-associated antigens (TAA) have been identified in the melanoma model which has allowed for immunization trials to evaluate therapeutic potential of tumor-specific T-cell induction. Some clinical trials reported limited success of T-cell mediated tumor rejection, reporting partial or complete regression in 10 to 30% of patients.[3] Although tumor regression was not observed following active immunization in vivo, ex vivo assays evaluating TAA-specific T cells demonstrated tumor recognition and subsequent T-cell activation suggesting that tumor-specific T-cell induction indeed occurs but alone is not adequate to induce tumor regression.[1] Recently, the usefulness and success of active-specific immunization (ASI) against TAAs as a means of eliciting a tumor-specific immune response leading to tumor regression and clearance has been a topic of debate and discussion.

Argument against Potential of Tumor Vaccination

Those unconvinced of the potential usefulness of cancer vaccines as a therapy argue that the clinical endpoint of ASI is tumor destruction and clinical trials have yet to successfully correlate ASI with clinical regression. Instead, other therapeutic methods should be investigated such as adoptive immunotherapy. In the viral model, vaccination shows no benefit to the host when administered during acute viral infection and Rosenberg et al[4] suggest that a similar phenomenon may be occurring during ASI against cancer and that cancer vaccines may be more useful if used as a preventative. In animal models, antitumor vaccinations were administered both in prophylactic and therapeutic settings. Prophylactic vaccinations against viral diseases and to prevent virally

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induced tumors were effective when synthetic peptide was used. However, nonvirally induced tumors did not respond to prophylactic vaccination, though antitumor efficacy was present. As a therapy, vaccination was effective in only limited number animal models.

Rather than measuring success based on immunologic data such as circulating TAA-specific T-lymphocytes, presence of tumor infiltrating lymphocytes (TILs) and histology, it has been suggested that success should be based on clinical regression following the Response Evaluation Criteria for Solid Tumors (RECIST) guidelines which require a 30% reduction in the sum of maximum lesion diameter and no novel or progressing lesions.[5-7] However, the more conventional criteria to characterize a clinical response are a "50% reduction in the sum of the products of the perpendicular diameters of all lesions without 25% growth of any lesion or the appearance of new lesions". Using this more common approach, Rosenberg et al[4] reported an objective response rate of only 2.6% in 440 patients following vaccine administration to patients with various types of metastatic cancer including melanoma, renal cell, ovarian, colorectal and breast, a rate which they found to be comparable to other vaccine trials. Patients were administered various vaccines such as peptide, viral vectors and naked DNA encoding tumor antigen. Although T cells activated against specific TAAs can be successfully generated in vivo, the lack of correlation to clinical response still exists. However, the report was biased by the aggregation of heterogeneous protocols into a single analysis and by the lack of mechanistic interpretation of the reasons for the lack of correlation between frequency of TAA-specific T cells and tumor regression.[8]

Various obstacles exist in the cancer ASI setting. One issue is that although T-lymphocytes capable of recognizing TAAs are generated, antigen recognition is not enough to mediate regression and T-cell mediated rejection of vascularized tumors.[3] In addition to recognizing TAAs, T cells must also be capable of localizing and surviving in target tissue. Another concern is that the number of circulating T cells may be inadequate to mount a clinical response. In adoptive therapy studies, it has been reported that between 5 and 75% of antitumor T cells are necessary to achieve somewhat successful clinical effectiveness.[9] Other important elements that should be addressed are the inability of tumor to activate quiescent or precursor lymphocytes, tolerance mechanisms including anergy,[3] suppressor influences by tumor or immune system[10,11] and tumor escape mechanisms.

Argument for Potential of Tumor Vaccination

Expectations from immunizations against cancer are similar to those from immunizations against pathogens. In the pathogenic immunization pathway an inflammatory response at the injection site activates monocytes making them capable of antigen uptake. The immunogen or adjuvant can cause monocyte maturation into professional antigen presenting cells (APCs) which migrate to loco regional lymph nodes where they interact with naïve and memory T cells, including those that can recognize the antigen and initiate the afferent loop of immunization. Differences between anti-cancer and anti-infectious ASI occur in the efferent arm. In a pathogenic model, after T cells are primed in the lymph nodes, they migrate back to the site of infection which is usually associated with inflammation allowing the T cells to become activated and to perform their cytolytic function. However, because tissue damage in cancer is typically not as extensive as in a pathogenic model, the tumor microenvironment is less conducive to producing inflammatory responses capable of stimulating tumor-specific T cells to perform effector functions. The afferent arm of immunization however, is functioning properly as demonstrated by identification of circulating antigen-specific T cells suggesting that the problem lies in effector function performance.[1]

Because T-cell induction does not equal clinical regression, certain aspects should be investigated such as effector function adequacy, frequency of immunogen-specific T cells, T-cell localization and function at tumor site, secondary stimuli and tumor escape mechanisms. The fact that tumor and tumor-specific circulating or intra-tumoral cytotoxic T-lymphocytes (CTLs) can coexist in the host suggests that there may be an issue with the adequacy of T-lymphocyte effector function. Following immunization, TAA-specific T cells typically express T-cell activation markers and secrete IFN-γ when stimulated ex vivo by cognate tumor. However, they do

not express perforin and other effector molecules and are small in size which is similar to that of resting T cells.[12,13] In addition, it is possible that TAA-specific T cells are not produced at a high enough frequency to induce tumor regression since there is evidence that in the virus model T-cell frequency directly correlates with disease clearance or resurgence. Tissue sensitivity to CTLs may also be variable depending on the tumor, which may determine the frequency necessary for regression. Mouse studies have shown that immune response intensity directly correlates with tumor regression[14] and that T-cell frequency directly correlates with number of immunizations administered. It has been reported that in order to reach a T-cell frequency comparable to that of acute infection, between 16 and 24 rounds of immunization might be necessary[15] and in most cancer vaccine trials only a few rounds of immunization are administered, which may limit the ability or success of the vaccine. Longer immunization schedules could be beneficial, albeit in a population not requiring urgent palliative or therapeutic intervention. Moreover, although T cells seem to have the ability to reach the tumor site, recognize antigen and produce interferon-γ (IFN-γ), they are not able to expand, nor limit tumor growth which is similar to the immune response in chronic viral infection in which CTLs are circulating but not eliminating virus.[16] It is possible that secondary stimuli such as interleukin-2 (IL-2) may be required in order to activate CTL effector function. Although tumor escape mechanisms offer an attractive explanation for the lack of correlation between TAA-induced T cells and clinical regression, we are inclined to believe that this is probably not the case and that it is instead a result of T cells that are not adequately stimulated for killing.

Although the RECIST guidelines are useful to determine tumor shrinkage, this may not be the best suited method to measure success in the cancer vaccination setting. In a literature review of clinical ASI studies Mocellin et al[8] report a response rate of tumor shrinkage in 10% of subjects, which would be even higher under RECIST guidelines however, this number does not correlate with clinical regression.[17] This demonstrates that contrasting "tumor response" from RECIST with "patient response" from increased survival following immunization may be more useful and that solely using the same RECIST criteria regardless of therapy, type of cancer and stage of disease may be dangerous. In clinical cancer vaccine trials, few have demonstrated robust responses satisfying RECIST guidelines, however prolonged survival has been observed as a measurable endpoint.[18]

Methods for Immune Monitoring following Active-Specific Immunization

Systemic Response

Developing a standardized method for immune monitoring of vaccine induced immune responses is of great importance for the development and evaluation of cancer vaccines. Standardization would allow vaccine study comparison between institutions however standardizing parameters and laboratory techniques among the large variety of cellular and molecular assays that are used to detect responses to vaccination is quite difficult.[19] A vast array of techniques and assays are regularly employed to monitor the systemic immune response following ASI. Furthermore, it should be emphasized that the human biology is the independent variable and relevant clinical parameters should be easily reproducible independent of the assays if they are truly associated with a particular determinism. Thus, excessive emphasis on assay validation and cross-validation may be unwarranted when the biology evaluated is not clearly relevant to disease outcome or the phenomenon studied is poorly linked to a clinical parameter.

Limiting Dilution Assays (LDA)

Two types of limiting dilution assays (LDAs) are typically used to measure systemic activation of circulating T cells, one measures antigen-specific T-cell proliferation and the other T-cell ability to lyse labeled tumor cells. Antigen-induced clonal expansion is detected via radiolabel incorporation into DNA to measure expansion of CTL and helper T cells. Briefly, cells are incubated for approximately 5 days in the presence of soluble antigen and ^{3}H-thymidine is added for several hours. DNA

synthesis, which is the first response of cells to the mitogenic potential of the antigen, is determined by measuring radioisotope incorporation associated with the cells. As a positive control, lectin PHA can be added as a nonspecific T-cell activator. This assay is extensively used because the desired outcome of any vaccination protocol is the expansion of an antigen-specific T-cell population.[20] The other assay measures the lytic ability of CTLs and helper T cells that are measured through radio-labeled tumor cells. This assay is important in immunization immunology because it is assumed that the ability to lyse and kill tumor targets in vitro is similar to CTL killing ability in vivo, however this has been difficult to prove. Tumor lysis can occur via two methods (1) CTL release of lytic granules containing perforin and granzymes causing pore formation in target membrane followed by lysis and (2) through the Fas-Fas ligand apoptotic pathway. The chromium release assay (CRA) elucidates CTL function by measuring the amount of ^{51}Chromium (Cr) released following lysis of labeled cells. Tumor target cells are labeled with ^{51}Cr and mixed with T-lymphocytes. Target cells spontaneously release ^{51}Cr slowly, so rapid ^{51}Cr release demonstrates target cell lysis. In a similar colorimetric assay, MTT tetrazolium salt is hydrolyzed by viable cells to form a blue crystal measurable in a microtiter plate reader. Additionally, fluorimetric methods including MUH and AlamarBlue give more sensitive results when compared to the CRA method. Fluorimetric assays are attractive because they avoid radioisotype use, however they do have longer assay times and require the purchase of a microplate reader.[20] From a clinical standpoint, CRA has often been used to monitor clinical immunization trials and to determine immunogenicity of tumor-related proteins however no correlation has been documented between antigen-specific proliferation and clinical outcome. In theory, vaccine-naïve patients with cancer or volunteers without cancer should not have detectable TAA-specific immunity. However, it has been reported in some cases that melanoma patients, as well as volunteers without cancer do have immune responses to melanoma differentiation antigens, making evaluation of the prevalence of immunity in the naive population important.[21,22]

Enzyme-Linked Immunospot Assays (ELISPOT)

The enzyme-linked immunospot assay (ELISPOT) is based on the ELISA principles and was originally established to detect antibody-secreting cells and was later adapted to detect antigen-specific T cells and T-cell frequency. Practically speaking, a 96-well nitrocellulose-bottomed microtiter plate is coated with an antibody that traps a specific cytokine. Peripheral blood mononuclear cells (PBMCs), isolated $CD8^+$ or $CD4^+$ lymphocytes are incubated in the wells in the presence of an antigen for 6 to 48 hours. If cells respond to antigen, they will release said cytokine which is then bound by the antibody in close proximity to T cell in the well. Cells are then washed from the wells to visualize cytokine release by T-lymphocytes by an enzyme-labeled detection antibody and its chromogenic substrate that attaches to the well surface. The final product consists of colored spots in the wells; each spot corresponds to one cell secreting the candidate cytokine. IFN-γ production is often used as a read out for T-cell activity because it is not typically spontaneously secreted in unstimulated T cells that occur with other cytokines, such as TNF-α in a small fraction of cells. Various studies have used ELISPOT to measure tumor-reactive T-lymphocytes in peripheral blood of patients with tumors and data suggest that the assay is capable of detecting low frequency T-cell responses.[20] One advantage of using ELISPOT over assays such as LDAs is that it does not rely on cell proliferation which better reflects individual IFN-γ producing cells and functional state in vivo.[19] Clinically, this method of immune monitoring has been useful in vaccination trials, most of which measured responses against peptides, melanoma cells, or idiotype protein in patients with myeloma.

Cytokine Flow Cytometry (CFC)

Cytokine flow cytometry (CFC) can be used to detect stimulated $CD4^+$ and $CD8^+$ T-lymphocytes at a low frequency following ex vivo stimulation with antigen by measuring intracellular IFN-γ as a surrogate of T-cell activation. CFC can be successfully performed with mononuclear cells obtained from PBMC,[23] whole blood,[24] lymph nodes or other biologic fluids.[2] Briefly, mononuclear cells are incubated for a total of 6 hours in presence of stimulating antigen to allow generation of high cytokine levels and for optimal cytokine staining. After 1 to 2 hours of stimulation, a cytokine secretion inhibitor such as brefeldin A is added to the culture. The cells

are then fixed at 6 hours post stimulation, permeabilized and stained with monoclonal antibodies that recognize both surface and intracellular proteins to be characterized via flow cytometric analysis. Due to the short stimulation time, problems associated with increased culture times such as apoptosis and proliferation need not be addressed. As we have previously shown[25-27] unstimulated lymphocytes and lymphocytes stimulated with irrelevant peptide do not exhibit cytokine secretion and thus background noise is rare. Moreover, super-antigens such as staphylococcal enterotoxin B can effectively stimulate a large proportion of lymphocytes as a positive control.[26] In studies investigating T-cell activation in response to stimulation with CMV and EBV viral epitopes, CFC was reliable in producing measureable amounts of IFN-γ.[25,26] Because tumor-specific T cells often produce less IFN-γ than virally stimulated T cells, they may be theoretically more difficult to detect using this method.[20,28] However, studies have demonstrated that CFC is in fact sensitive enough to detect immune responses to tumor antigens in spite of the fact that IFN-γ frequencies are lower than when compared to infection.[29] Others report the need for in vitro sensitization for frozen and thawed samples, rather than direct ex vivo testing.[30] In addition to immune monitoring, CFC may also be a powerful tool in vaccine development by identifying novel TAA capable of eliciting immune responses.

Tetramer Analysis with Soluble Major Histocompatibility Complex (MHC)/ Peptide Complexes

Soluble MHC/peptide tetramers can be produced that are conjugated to a fluorochrome and stably bind to a specific T-cell receptor (TCR). Tetramers can be generated for MHC class I $CD8^+$ T-cell screening as well as for MHC class II $CD4^+$ T-cell screening. Fluorescent MHC/peptide tetramers when incubated with a heterogeneous population of T cells will bind those T cells expressing MHC/peptide-specific TCRs which can then be detected by flow cytometric assays. This tool is useful in identification of antigen-specific $CD8^+$ and $CD4^+$ T cells in a polyclonal T-cell population and to generate information on T-cell functionality when combined with additional assays.[19,20] Methodology for tetramer generation has been established and described.[31-33] Tetramer analysis following vaccination has numerous advantages over some other methods. Tetramers allow cell enumeration without employing indirect functional assays in vitro and also allows cell sorting to isolate antigen-specific T cells which provides a source for TCR analysis and for cells targeted to adoptive transfer therapy. In addition, cellular phenotype can be obtained by using markers for activation status, costimulatory receptors, homing and others, while simultaneously staining for intracellular proteins to study T-cell stages of those responding to vaccination.[19] Although tetramers are a powerful tool, they do have some limitations such as that MHC/peptide tetramers bind TCR with minimal avidity, which may allow some T cells of functional and clinical importance to be overlooked. In addition, some clinically important epitopes bind MHC with low affinity eliminating the possibility to produce an effective tetramer complex.

Quantitative Reverse Transcription-Polymerase Chain Reaction

Originally, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was developed to measure viral loads in patients and for monitoring viral infection following transplantation. Investigators at the National Cancer Institute Surgery Branch adapted this procedure for use in evaluating T-cell activation by measuring IFN-γ transcript levels in melanoma patients following ASI with peptide. Kammula et al[34,35] compared IFN-γ transcript levels in PBMC pre and postvaccination as well as in PBMC stimulated ex vivo with the relevant epitope. Data from this study found that the qRT-PCR results correlated well with results from HLA/peptide tetrameric complexes and intracellular CFC. This assay allows the investigation of gene expression of an unlimited number of genes from small samples, such as FNAs, from a likewise minimal amount of candidate RNA. Because this is a sequence-based method, it also allows for investigation of any gene with a known sequence. Additionally, after cDNA is generated from the RNA of clinical samples, it can be stored safely for a long time for future analyses. This method also provides flexibility in that it can be applied to T-cell reactivity to whole proteins, protein mixtures, whole tumor cells without first determining relevant peptides/HLA restrictions.[20]

Transcriptional Analysis of Circulating T Cells Following Active-Specific Immunization

In a transgenic mouse model Kaech et al[12] characterized time-dependent phenotypes of $CD8^+$ T cells following acute exposure to antigen. One week following antigen exposure, expanding $CD8^+$ T cells were found to be cytotoxic when tested ex vivo and had a transcriptional profile rich in effector function. In the following two weeks during the contraction phase, a memory phenotype was observed that responded to cognate stimulus measured by IFN-γ secretion, but was not capable of cytolytic activity and other effector functions. These findings correlate well with the TAA-specific immunization model in which a time-limited course of TAA-exposure is followed by a rest period of a few weeks. In this model, immunization induced T cells retain the effector phenotype ($CD27^-$, $CCR7^-CD45RA^{high}$) and IFN-γ responsiveness, however they cannot exert effector functions.[1,36] Monsurró et al[13] described this 'quiescent' phenotype through transcriptional profiling of an immunization-induced T-cell population not capable of exerting ex vivo cytotoxicity that was found to lack gene expression associated with T-cell activation, proliferation and effector function. This study was important because it demonstrated the significance of evaluating the functional status of vaccine-induced T cells at the global level pointing out that circulating T cells induced by vaccines do not have a phenotype of true effector cells and this finding may provide the most likely explanation for the lack of correlation between TAA-specific T-cell frequency in the blood and tumor regression.[1] Importantly, this study also demonstrated that transcriptional analysis of rare sub-populations of T cells can be performed using sorting procedures; further improvement in sorting technologies using high-speed FACS sorters has increased the yield and purity of such subsets allowing sophisticated distinctions among various circulating lymphocytes and subtleties about their interactions.[27,37]

Tumor-Site Response, Tumor Microenvironment

The Immune Surveillance Hypothesis

Immune surveillance, as described by Wang et al is a hypothesis that may be useful in understanding spontaneous cancer rejection.[38] Although no direct way exists to test this hypothesis, it has been suggested that the immune system is in a constant battle in surveillance against neoplastic development.[39] Examples of spontaneous rejection that drove the hypothesis include Rosenberg's patient with gastric cancer who was found to be disease free years later[40] and studies that demonstrated a reduction in size or complete regression of renal cancer pulmonary metastases following primary tumor removal.[41-43] In these instances, it is suggested that perhaps through immune surveillance, the host responded to a systemic presence of cancer. These occurrences support experimental evidence that demonstrates an increased prevalence of cancer in mice that lack immune effector mechanisms such as IFN-γ production or are deficient in T-cell function.[44] Remarkable human examples occur within Epstein-Barr Virus (EBV)-induced lymphomas that are often observed during immunosuppression. These lymphomas are readily reversed both when immunosuppression is discontinued and when EBV-specific CTL are adoptively transferred to the patient.[38] Thus, there is sufficient evidence that innate and adaptive immune responses play a role in the modulation of the growth of at least a subset of cancers.

Transcriptional Profiling Immune Responses against Tumors

Although many studies have focused on measuring immune responsiveness by way of circulating peripheral T cells, it is also important to study the immune responses occurring in the target organ and in the tumor microenvironment. Studying the tumor microenvironment is relevant as it has been shown that cancer cells can significantly affect the surrounding environment,[45] some cancers are more sensitive to immune responses than others,[46-48] immune responses for particular cancers may be predetermined[49] and it may provide insight into the effects of IL-2 on microenvironment.[2,16,50-53] For example, the importance of tumor microenvironment has been observed during the evaluation of tumor-free peritoneum in patients with epithelial ovarian

cancer. Alterations in surrounding tissue were observed that seemed to be a result of soluble factor secretion from tumor deposits that activated cell-cell interactions and adhesions as well as extra-cellular matrix modulation and growth.[48] Use of transcriptional profiling techniques on tumors as well on as surrounding tissues may be important in providing insight into the intricacies of tumor growth and persistence.

Use of transcriptional analysis at the level of tumor microenvironment has proven to be valuable. Initially, from transcriptional analysis of frozen tissue and cell lines, melanomas were thought to segregate into two molecular subclasses.[54] However, additional transcript analysis of melanoma lesions sampled by repeated FNAs have shown that what were thought to be subclasses are probably two phases of an evolving process that eventually leads to loss of gene expression associated with melanoma ontogeny.[49] As such, transcriptional analysis is beneficial in evaluating effects of ontogeny of molecular sub-classifications. This approach was used to compare profiles of normal kidney samples and primary cancers of varying histology to renal cell cancer (RCC) profiles and confirmed that the molecular basis of the subclasses correlates to the level of differentiation of individual cancers. When the genes that were co-expressed by normal kidney tissue were removed from analysis, RCC displayed the same profile as other cancers, demonstrating similarities in oncogenic processes.[48] In fact, use of transcriptional profiling allowed Wang et al[46] to elucidate molecular signatures for melanoma in which most melanoma-restricted immune-associated genes cluster tightly together including those genes associated with natural killer (NK) cell and activated $CD8^+$ T-cell function. Moreover, a large cluster of genes is shared between melanoma and RCC; although the significance of these similarities should be investigated further.

IL-2 has been credited with inducing regression of both melanoma and metastatic RCC and is also thought to play a role in immune-mediated cancer regression. Because of this capability, Panelli et al investigated the transcriptional profile of FNA obtained from melanoma metastases before and during IL-2 therapy. In this study, it was observed that IL-2 does not cause migration, activation, or proliferation of T cells at tumor site, it does however induce a cytokine storm that is surged by monocytes and NK cells, mimicking acute inflammation. Monocytes and NK cells contribute to immune response by destroying cancer cells and taking up shed TAAs that are then presented to adaptive immune cells.[50] The transcriptional analysis of a lesion responding to IL-2 demonstrated gene activation that overlapped with those genes identified in the profile of TAA-specific T-cell activation in vitro.[13] It is likely that IL-2 does not directly alter the tumor microenvironment, but that alterations are dependent on the downstream production of immune modulators by IL-2 stimulated cells, which then affect the microenvironment.[51,55] In order to investigate the potential effect of the cytokine storm on intra-tumoral mononuclear phagocytes Wang et al[36] analyzed the profile of target cells following stimulation with panel of cytokines and identified two major cytokine classes capable of inducing classical and alternative mononuclear phagocyte activation. In summary, the study of circulating T-cell responses needs to be complemented by the study of functional signatures within the tumor microenvironment at time points relevant to their function. In particular, the dynamic phase of the immune response in which a switch is observed between a chronic inflammatory process conducive to cancer growth onto an acute one leading to cancer destruction needs to be studied by comparing circulating and peripheral immune responses.[2] Following this strategy we have recently proposed a model representative of this dynamic phase of the immune response which is relevant not only to tumor rejection but, more generally, to immune-mediated tissue-destruction in the context of allograft rejection, pathogen clearance and autoimmunity; we called this model "the immunologic constant of rejection"[16] (see also next section). The immunologic constant of rejection predicts that tissue-specific destruction in mediated to activation of Type II interferon signatures inclusive of CXCL-9 to -11 and CCL5 chemokines, activation of cytotoxic T cells and Natural Killer cells with their localization, expansion and activation at the tumor site leading to high levels of expression of immune effector genes such as granzyme A and B, Perforin and FAS. Thus, immune responses switch during immune rejection from a quiescent circulating phenotype onto an activated effector natural-killer cell type within the target organ.[1,16]

Conclusion

Although investigation into T-cell responses to cancer vaccines is far from complete, great progress has been made to begin understanding the complex interactions leading to cancer regression. Although vaccination against TAAs increases antigen-specific T cells in peripheral blood, this increase is not correlated with clinical regression, which sparked investigation into T-cell function following immunization. One explanation that may describe this phenomenon is immunoediting as a means of tumor escape from immune recognition.[56-58] Alternatively, we and others have hypothesized that although tumor-specific T cells are induced by vaccination, their function is inadequate for tumor regression.[59-61] This lack of function may result from various problems during tumor-host interactions including inadequate T-cell receptor (TCR) engagement with epitope, insufficient host costimulation, lack of T-cell localization to target tissue and the complexity of tumor-host interactions in the tumor environment resulting from varying tumor phenotypes and the immune mediators secreted into the microenvironment.[60] In order to determine functional and genetic differences, Monsurró et al[13] compared an antigen-specific subset of T-lymphocytes to properly functioning T-lymphocytes. The immunization-induced subset of cells was described as having a quiescent effector phenotype lacking proliferative and cytotoxic capabilities ex vivo. This phenotype was also characterized by down-regulation of genes important in T-cell activation, proliferation and effector function. In one clinical study, patients with Stage I-III melanoma were vaccinated with a modified gp100 peptide. Although in the majority of patients vaccinated induced high avidity, tumor-specific T cells, they were still found to be of low function in tumor lysis assays.[62] Moreover, Chen et al[63] developed a high throughput array method using peptide/MHC complexes with antibodies against secreted factors to capture T-lymphocyte secreted cytokines. This methodology is useful for characterization of antigen-specific $CD8^+$ T-lymphocyte functionality in clinical samples following vaccination and may be useful in correlating lymphocyte function to clinical outcome. The clinical samples from ten melanoma patients vaccinated with a gp100 peptide evaluated in this study displayed distinct differences in cytokine secretion profiles both in patient-specific and antigen-specific $CD8^+$ lymphocytes, demonstrating the variability in T-cell function following vaccination.

Another point that has been elucidated in recent years is that TAA-specific T cells must not only be induced following immunization, they must be active and functioning at the tumor site. We propose that T-cell function and ultimately tissue destruction in cancer may occur through a route similar to other pathological processes such as infection, allograft rejection and autoimmunity and suggest that an "immunological constant of rejection" may exist as the common mechanism for these disease processes. Transcriptional profiling studies revealed that this immunological constant includes activation of interferon stimulated genes (ISGs) and immune effector functions (IEFs).[16] Sarwal et al[64] studied the basis of acute rejection in kidney allografts and identified ISGs, granzymes, B and T-cell signature. Similarly, studies regarding immune-mediated melanoma metastasis rejection during IL-2 therapy demonstrated activation of ISGs, granzymes as well as transcripts for activated CTL and NK cells.[49,50,52] Based on these results, we hypothesized that the last step in the pathway leading to cancer rejection is broad activation of cytotoxic mechanisms by innate or adaptive immune cells. This hypothesis was studied in the Imiquimod-mediated rejection of basal cell carcinoma (BCC), in which rejection was associated with expression of ISGs, IEFs, IFN-α, IFN-γ and infiltration of CTL and NK cells, with a complete lack of B-cell involvement.[52] These studies, among others, have demonstrated the association of Type I pro-inflammatory modulators, especially IFN-α and IFN-γ, with tissue-specific destruction. Because this activation is present in many chronic inflammatory conditions, it alone is unlikely to be adequate for tumor or tissue rejection, but in combination with additional immune responders may recruit CTLs and initiate a cascade leading to rejection.[16]

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Chapter 15

Memory T-Cell Homeostasis and Senescence during Aging

Sian M. Henson* and Arne N. Akbar

Abstract

Evidence is accumulating that old individuals are more susceptible to infection with organisms to which they were previously immune, indicating that there might be a limit to the persistence of immune memory. The prevailing concept is that defects in memory T-cell populations result from inexorable end-stage differentiation as a result of repeated lifelong antigenic challenge. We discuss here mechanisms that might constrain the persistence of memory T cells and consider whether humans will suffer from memory T-cell exhaustion as life expectancy increases.

Introduction

The immune system undergoes dramatic restructuring with age, leading to a decline in immune responses and an increased vulnerability of old individuals. The incidence and severity of infectious diseases, such as pneumonia,[1] meningitis,[2] sepsis,[3] urinary tract infections,[4] infection with respiratory syncytial virus[5] or influenza[6] all increase with age. Indeed the mortality rate of older adults suffering urinary tract infections or tuberculosis is ten-fold higher than that of young adults.[7] This waning immunity in old age results from defects in numerous different leucocyte populations. However the dysfunction is most pronounced in T cells, as old individuals often experience reactivation of latent organisms, such as varicella zoster virus (VZV)[8] and occasionally Epstein Barr virus (EBV),[9] mycobacteria[10] and cytomegalovirus (CMV).[11] The VZV reactivation that is observed in old individuals is associated with a decrease in VZV-specific T-cell numbers but not antibody levels, indicating that the immune defect might be at the T cell but not the B cell level.[12] This T-cell immune decline is marked by a dramatic decline in the number of naïve T cells as a result of thymic atrophy.[13,14] This reduced thymic output leads to the peripheral expansion of naïve and memory T cells to regenerate the T-cell pool, which in turn leads to the accumulation of oligoclonally expanded, functionally impaired T cells.[15,16] These age associated changes contribute to the inability of the aged immune system to respond to new antigenic challenge and mount poor responds following vaccination.[17]

Phenotypic and Functional Differentiation of T Cells during Aging

T-cell memory is lost during aging however it is not clear whether this is a qualitative or quantitative defect. The human memory T-cell pool is not homogeneous but contains a multitude of cells that are specific for different antigens. Reports have compared the relative number of memory T cells in young and old individuals. Data shows older adults to have significantly higher numbers

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Table 1. Phenotypic and functional characteristics of human T-cell subsets

Phenotype	Naïve	Central Memory	Effector Memory	CD45RA Memory	References
CD45RA	+++	–	–	+++	86,87
CD45RB	+++	+++	+	+	86,87
CD45RO	–	+++	+++	–	86,87
CD28	+++	++	+/–	+/–	86,88
CD27	+++	++	+/–	+/-	86,87
CCR7	+++	++	–	–	86
CD62L	+++	+++	+	+	86
LFA1	–	+++	+++	+++	45
CD95	–	+++	+++	+++	89,90
CTLA-4	+++	++	++	+	91,92
PD-1	+	+++	+++	++	37,93
KLRG1	+	++	++	+++	19,32
BCL-2	+++	++	+	++	45,89,90
Telomere length	+++	++	+	++	27,45

Following antigen stimulation naïve T cells lose expression of CD45RA and become CD45RO$^+$. Upon differentiation to an effector memory population T cells lose CD45RB, CCR7, CD62L, CD28, CD27 and CTLA-4, while expression of LFA1, CD95, PD-1 and KLRG1 increase. In young adults, these CD45RA$^+$ revertant T cells have similar telomere lengths to the central memory pool and do not require proliferation to mediate effector function. While in old adults these cells are highly differentiated and close to replicative senescence.

of T cells that are specific for persistent viruses such as EBV and CMV, but these cells have a decreased capacity to secrete interferon-γ (IFNγ).[18-20] Implying that as we age virus-specific memory T cells become less effective in the suppression of viral replication.[19,20]

There are numerous reports cataloging the phenotypic and functional characteristics of human T cells to identify qualitative changes that occur during aging (Table 1). Old individuals show an increased proportion of T cells that are highly differentiated, with similar phenotypic changes occurring in both CD4$^+$ and CD8$^+$ T cells during differentiation. However the rate at which these changes happen varies within each subset, with age-related changes being more pronounced on CD8$^+$ T cells due to a greater homeostatic stability of CD4$^+$ T cells.[21-23] Highly differentiated T cells are characterized by the loss of the cell surface costimulatory molecules CD27 and CD28, CD8$^+$ T cells losing CD28 first followed by CD27 with the converse being true for CD4$^+$ T cells.[24-27] Initially, it was thought that the loss of CD28 was a major factor in the reduced activation and function of these cells,[28] however, it has been shown that there is considerable redundancy in costimulatory receptor usage in highly differentiated T cells and that alternative receptors, such as 4-1BB may be engaged to promote T-cell activation in CD28$^-$CD8$^+$populations.[29,30]

In addition to the loss of costimulatory receptors inhibitory receptor expression increases further adding to T-cell dysfunction during aging. We and others have shown an age related increase in the expression of the inhibitory receptor killer cell lectin-like receptor G1 (KLRG1) on CD4$^+$ and CD8$^+$ T cells,[20,31] with expression being highest on highly differentiated CD28$^-$CD27$^-$ T cells. KLRG1$^+$ T cells are unable to undergo clonal expansion, even in the CD28$^+$ subset,[31] furthermore KLRG1 expression correlates with the inability to proliferate upon stimulation.[32] The ligands for

KLRG1 have recently been identified[33] and it has now become possible to block KLRG1 signalling, resulting in increased proliferation in both $CD4^+$ and $CD8^+$ T cells (Henson et al in press). Offering the possibility of increasing the proliferative capacity of highly differentiated T cells.

The inhibitory B7 family member Cytotoxic T-lymphocyte antigen 4 (CTLA-4) also increases with age,[34,35] with the largest change in CTLA-4 expression in human $CD8^+$ T cells being found in the $CD28^+$ subset (Henson et al unpublished data). This increased expression of CTLA-4 with age does not occur at the transcriptional level, for we and others have demonstrated that CTLA-4 mRNA does not to differ with respect to age in either $CD4^+$ or $CD8^+$ T cells.[23,36] We believe the elevated CTLA-4 expression arises from a change in the recycling rate of CTLA-4 from the cell surface.

There has been much interest in the inhibitory receptor program death 1 (PD-1), as blockade of PD-1 signalling has been shown by numerous groups to boost immune responses.[37,38] We and others have found the expression of PD-1 on $CD4^+$ and $CD8^+$ T cells does not change with age at the RNA or protein level.[23,36] The expression of PD-1 is not dependent on age but is dependent on viral status and our data is in concordance with the idea that PD-1 serves as a marker on viral specific $CD8^+$ T cells to indicate the degree of T-cell exhaustion.[39] The level of PD-1 protein per cell is important in regulating T-cell dysfunction, for example CMV positive old donors express more PD-1 and have a higher degree of $CD8^+$ T-cell dysfunction than donors who are CMV negative (Henson et al unpublished data).

Highly differentiated $CD28^-CD27^-CD4^+$ and $CD8^+$ T cells re-express CD45RA. These CD45RA re-expressing or revertant T cells accumulate with age,[25,40] with $CD4^+$ revertant T cells being found in lower numbers.[24] $CD8^+$ revertant T cells are thought to arise from the less differentiated central memory population through interleukin-15 (IL-15) driven homeostatic proliferation in young individuals.[40,41] These cells do not require proliferation to mediate potent effector function and are resistant to apoptosis.[40-42] Nevertheless, under appropriate conditions, these cells can be induced to proliferate,[42,43] suggesting that revertant cells are an effector population that has been functionally reprogrammed away from proliferation. This theory may not wholly apply to old individuals, as revertant T cells function poorly suggesting that during the course of aging, these populations are eventually driven to end-stage differentiation.[27]

Telomere Erosion and T-Cell Memory

After a finite number of divisions T cells reach replicative senescence,[44,45] which is the closest approximation to a functional definition of end-stage differentiation in T cells. The sensing mechanism or 'mitotic clock' that is responsible for setting a limit on proliferative lifespan is the telomere.[46] The telomere is a repeating hexameric sequence of nucleotides that is found at the ends of chromosomes.[47,48] Each division of a cell leads to the loss of 50-100 base pairs of telomeric DNA owing to the inability of DNA polymerase to fully replicate the ends of chromosomes.[46,47] Marked shortening of telomeres causes chromosomal instability and results in end-to-end fusions.[49] In turn, this triggers DNA damage-repair programmes through the activation of p53 and results in growth arrest and/or apoptosis.[50]

The measurement of telomere length has been used to assess the extent of differentiation in T cells, with both $CD4^+$ and $CD8^+$ T cells in old individuals have significantly shorter telomeres than the same cells from young individuals.[25,27] The rate of telomere loss is retarded by the enzyme telomerase, a RNA-dependent DNA polymerase, that synthesizes telomeric repeats maintaining telomeres during cell replication.[51] This enzyme complex consists of a catalytic reverse transcriptase protein, telomerase reverse transcriptase (TERT), a RNA template and a number of associated proteins.[47] Telomerase activity after activation was found to be highest in undifferentiated T cells, lower in the intermediate and very low in the highly differentiated $CD28^-CD27^-$ T-cell populations.[25,27,52] Suggesting that the ability to induce this enzyme is lost as T cells differentiate progressively. Thus, highly differentiated effector T cells have short telomeres and a limited replicative lifespan.[25,27]

Telomerase Regulation in Differentiated T Cells

Little is know about the mechanism for the decrease in telomerase activity during T-cell differentiation. Although resting human $CD4^{+}$[53] and $CD8^{+}$ T-cell populations[54] do not express telomerase activity without activation, they express hTERT,[30,53] indicating that activity of the enzyme is not only regulated at the level of protein expression. Signals via the T-cell receptor (TCR) and also costimulatory molecules such as CD28 are required for the induction of telomerase activity, which peaks after 4-5 days of stimulation and then decreases to baseline by 10 days.[27,55,56] As T cells are repeatedly activated in vitro, both the peak and duration of telomerase activity are decreased[27] and highly differentiated $CD28^{-}CD27^{-}$ T cells activated in vitro express low telomerase activity at all time points measured.[27]

The induction of telomerase activity is associated with an increase in hTERT protein expression,[57] however it is not the net hTERT protein increase but the phosphorylation and translocation of hTERT from cytoplasm to nucleus that regulates telomerase function.[53] hTERT is a substrate of the kinase Akt, which itself requires phosphorylation at two different sites for activity.[58] We have shown there to be a specific defect in Akt phosphorylation at the Ser^{473}Akt site in the highly differentiated $CD8^{+}CD28^{-}CD27^{-}$ T-cell subset but not in less differentiated populations.[30] Collectively, these results suggest that telomerase down-regulation in highly differentiated T cells is not only achieved by transcriptional control but is also related to changes in posttranslational modification of this enzyme.

Phosphoinositide 3-kinase (PI3K) dependent Akt activation can be regulated through the tumor suppressor phosphatase and tensin homolog (PTEN).[59] PTEN acts as a phosphatase to dephosphorylate phosphatidylinositol (3,4,5)-trisphosphate (PIP3) back to phosphatidylinositol (4,5)-bisphosphate,(PIP2) removing the membrane-localization factor from the Akt complex, decreasing the rate of Akt activation.[60,61] PIP3 can also be dephosphorylated by the SH2 domain-containing inositol phosphatase (SHIP), causing the inhibition of Akt by again regulating Akt's membrane localization.[62] The src homology 2-containing protein tyrosine phosphatase-1 (SHP-1) is also thought to have a negative role on Akt activity dephosphorylating the p85 subunit of PI3K.[63] However it has been shown to dephosphorylate PTEN and hence potentiate PI3K activity.[64] SHP-2 also appears to have a convoluted role in Akt regulation, with data showing it to be required for PI3K activation,[65] whereas other reports indicate that SHP-2 interferes with PI3K activation.[66]

We have investigated whether telomerase activity in highly differentiated T cells can be upregulated through the interruption of inhibitory receptor signaling, notably PD-1 and KLRG1 which signal via SHP-2 and SHIP respectively.[67,68] Whilst we can restore the defect in phosphorylation of Ser^{473}Akt in highly differentiated $CD28^{-}CD27^{-}$ $CD8^{+}$ T cells, we see no increase in telomerase activity per proliferating cell using blocking antibodies directed to the ligands of PD-1 and KLRG1 (Henson et al in press), suggesting that telomerase activity is not controlled by Akt in primary $CD8^{+}$ T cells. A recent paper has demonstrated an increase in telomerase activity following blockade of the PD-1/PD-L1 pathway in $CD8^{+}$ T cells using HIV peptides,[69] however the authors don't take into account the increased proliferation caused by interrupting PD-1 signaling.

Telomerase activity can also be controlled by the cytokines IL-7 and IL-15. While IL-7 is important in the homeostatic regulation of the $CD4^{+}$ T-cell pool, IL-15 regulates homeostatic expansion of both naïve[70] and memory $CD8^{+}$ T cells.[71,72] These cytokines have been shown to induce telomerase in both $CD4^{+}$ and $CD8^{+}$ adult T-cells[70,71] but not in cord blood T-cell populations.[73] The exact signaling pathways by which this occurs is not clear; however, it has been shown that IL-15 acts via a Jak3 and PI3K pathway to induce telomerase activity.[71] Indeed, we have shown that IL-15 only partially restores the low telomerase induction in highly differentiated $CD8^{+}CD28^{-}CD27^{-}$ T cells.[30]

Loss of T-Cell Memory during Aging

Highly differentiated T cells accumulate with age as a result of continuous antigen-induced turnover of populations that are specific for frequently encountered antigens. CMV-specific T cells

are more differentiated than those specific for VZV, EBV, herpes simplex virus (HSV), influenza virus and also tuberculin-purified protein derivative (PPD).[25] This may be due in part to the effects of IFN-α, known to inhibit telomerase,[74] induced by the triggering of plasmacytoid dendritic cells by CMV.[25] CMV infection has been considered harmless to individuals with a functional immune system. Longitudinal studies, however, have defined an immune risk phenotype (IRP) in healthy old individuals, which is predictive of significantly decreased two and four year survival of patients above the age of 80.[18,75] The IRP phenotype is composed of a cluster of immune parameters including CMV seropositivity, a CD4:CD8 T-cell ratio of <1 due to increased $CD8^+$ T cells, an increased proportion of highly differentiated $CD8^+CD28^-$ T cells, the presence of $CD8^+$ T-cell clonal expansions and elevated serum levels of pro-inflammatory cytokines.[18,75] There is evidence that all these changes may be primarily due to the effects of persistent infection with CMV in old adults subjects and this has been reviewed extensively elsewhere.[76] Thus, CMV might have a more insidious effect on the immune system than previously appreciated; however, it is unclear how the various immune changes that comprise the IRP are linked and why CMV infection in particular appears to reduce the survival of old adults in the IRP group.

Another feature of the T-cell compartment in old individuals is the number of large clonal populations indicating that cells approaching immunosenescence might accumulate rather than disappear. Many of these clones are specific for antigens that are continuously present, such as CMV and EBV.[19,20,75] One explanation for this paradoxical observation is that clonal evolution occurs during persistent viral infection,[77] which drives the specific T-cell clones with the highest avidity and/or functional activity to replicative exhaustion (Fig. 1). These may be replaced by other populations of less efficient cells in the memory pool.[77-79] This hypothesis is consistent with the observation that the expanded, highly differentiated CMV-specific $CD8^+$ T cells that are found in old subjects have decreased functional activity.[80] This accumulation of suboptimal highly differentiated CMV-specific T cells causes overcrowding of the memory T-cell pool, leading to the constriction and loss of memory T-cell populations.[80,81] For example, infrequently present memory T cells such as vaccinia virus and influenza virus[82] or T cells that are usually inaccessible to the

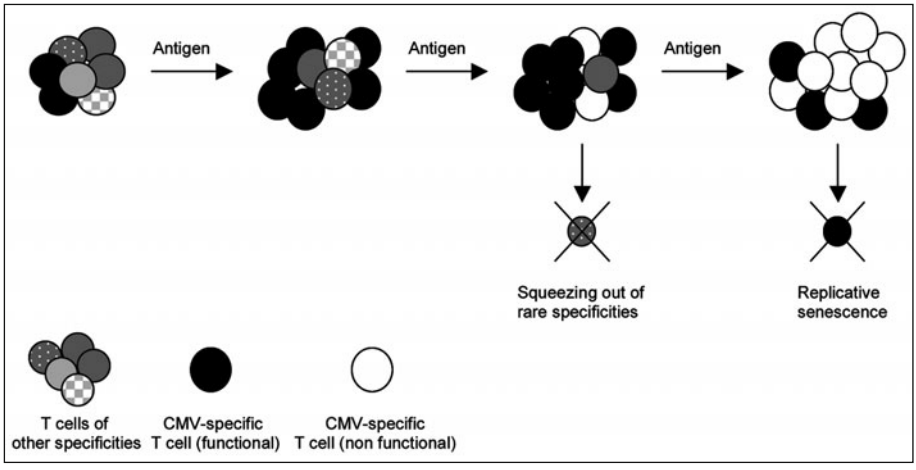

Figure 1. Effect of CMV-specific T-cell differentiation and accumulation. CMV-specific T cells are present at a relatively high frequency compared with cells of other specificities. Following antigen encounter, CMV-specific cells with the highest efficiency will expand preferentially. Large expanded CMV-specific clones reduce the available immunological space for T cells of other specificities. Leading to the disappearance of memory T-cell populations that are specific for infrequently encountered antigens. As the efficiency of available antigen-specific cells decreases, bigger expansions will be necessary to control the virus. This will eventually lead to large accumulations of nonfunctional CMV clones.

immune system such as HSV and VZV.[83] The most severe manifestation of antigen-specific T-cell exhaustion occurs when the suboptimal T cells are lost through replicative senescence. When this occurs, the prediction would be that CMV-specific T cells would diminish in number and an increased incidence of CMV-mediated disease would be reported. However, this has not been observed in old aduls thus far but cannot be ruled out in the future as life expectancy continues to increase.[84] This suggests that it may be important to consider whether strategies targeting CMV replication such as anti-viral therapy or anti-CMV vaccination may be used to preserve immune function during aging.[85]

Conclusion

Human life expectancy has doubled in the last 150 years and continues to increase, meaning that memory T-cell populations will also need to persist for longer in the future. We have highlighted that T-cell memory is lost during aging increasing the susceptibility of old individuals to infection by organisms to which they were previously immune. This loss of T-cell memory arises from antigen-driven differentiation and telomere erosion. Although telomere erosion has been viewed as a crucial mechanism that safeguards against malignant transformation and proliferation of T cells, the gradual loss of T-cell memory might be the price exacted for this protection. We suggest that prevention of chronic antigenic stimulation by prophylactic vaccination might be the most effective strategy to prevent declining immune competence with age.

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