CHAPTER 3

The Expansion and Maintenance of Antigen-Selected CD8$^+$ T Cell Clones

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Contents

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Abstract

The biological purpose of the mature, postthymic $CD8^+$ T cell is to respond to microbial antigens with a developmental program of clonal expansion and concomitant differentiation leading to effector cells (T_{EFF}) that provide antimicrobial defense. Because many microbial infections persist into a chronic phase, this antigen-stimulated developmental program must be capable of continually generating T_{EFF}, perhaps for the lifetime of the individual. This chapter proposes that the ability of a $CD8^+$ T cell clone to maintain the continual production of T_{EFF} during periods of persistent antigenic stimulation is based on a program that has two sequential phases of clonal expansion: an initial stage that occurs mainly in the secondary lymphoid tissues and is mediated by ligation of the T cell receptor (TCR) and CD27, and a subsequent, IL-2-dependent phase that occurs predominantly in peripheral, nonlymphoid tissues. The TCR/CD27-dependent phase establishes a nondifferentiating, self-renewing pool of clonally expanding cells, and the IL-2-dependent phase mediates continued clonal expansion that is coupled to the development of T_{EFF}. The two pools are linked by the process of asymmetrical division within the self-renewing subset so that, at steady state of cellular replication in this TCR/CD27-dependent subset, one daughter cell remains undifferentiated and the other initiates its commitment to IL-2-dependent terminal differentiation. Superimposed on this basic scheme are a shift in the $CD8^+$ T cell response to type I and II interferon (IFN) from anti- to pro-proliferative and transcriptional control of replicative senescence by Bmi-1, Blimp-1, and BCL6/BCL6b. This developmental program

ensures that despite the occurrence of cellular senescence antiviral CD8$^+$ T cell clones are maintained for the duration of persistent viral infections.

1. BACKGROUND

1.1. Introduction

Of the two biological challenges for the CD8$^+$ T cell, maintaining the continuous production of effector cells (T_{EFF}) during persistent infections and preserving clones that have been selected during primary infections for enhanced responses to subsequent infections, immunologists have tended to focus their efforts on explaining the basis for the latter capability, perhaps because it holds the promise of leading to improved vaccines. However, when considering whether it might be more detrimental to the individual if the CD8$^+$ T cell system could not continually produce T_{EFF} during persistent infections or could not maintain antigen-selected clones after a primary infection, it is clear that the former circumstance would be disastrous, whereas the latter may or may not be, depending on whether a secondary infection occurs. Some have even argued that surviving the primary infection indicates that a primary CD8$^+$ T cell response for that microorganism is sufficient. Furthermore, as immunological control of persistent viral infections is often mainly mediated by CD8$^+$ T cells while control of secondary viral infections that lack a latent or persistent phase is often based on the humoral response, the ability of the CD8$^+$ T cell system to produce continually T_{EFF} may be more biologically important than is its ability to persist in an apparently quiescent, yet alert state between acute infections. Understanding the signals that mediate continual replication of CD8$^+$ T cells *in vivo* without clonal senescence, if this is the mechanism for long-term CD8$^+$ T cell control of persistent viral infections, is not only inherently interesting but also, by defining the rules for maintaining the replicative function of the antigen-experienced CD8$^+$ T cell, it may lead to strategies for improved adoptive CD8$^+$ T cell immunotherapy. Therefore, the purpose of this chapter is to present an interpretation of the literature in relation to the developmental program that enables the CD8$^+$ T cell system to cope with persistent microbial infections.

With this being the intention of the chapter, it is necessary to discuss briefly the nomenclature used by investigators in the field of CD8$^+$ T cell biology. Despite the less compelling role for the CD8$^+$ T cell system in classical immunological memory, which is the ability to respond to a second infection more effectively than to the first, the nomenclature of the antigen-dependent phase of CD8$^+$ T cell development is dominated

by memory terminology. All $CD8^+$ T cells generated during antigen-dependent development, between the naive, antigen-inexperienced cell, and the terminally differentiated cell, are referred to as memory cells, even in the context of persistent infections where such terminology is probably inappropriate. This usage is too common to change now, but by defining the functional characteristics of each ''memory'' subset, the potential role of that subset in persistent, as well as repetitive, infections will become evident.

1.2. Subsets of antigen-selected $CD8^+$ T cells: Central memory, effector memory, and effector cells

In 1999, Lanzavecchia and his colleagues (Sallusto *et al.*, 1999) reported that four subsets of human peripheral blood $CD8^+$ T cells could be distinguished when assessed for the expression of CD45RA and the chemokine receptor, CCR7. These were

1. A $CD45RA^+$, $CCR7^+$ subset, which was considered to be composed of naive cells because they had the naive CD45 isoform and lacked potential effector functions, such as the capacity for rapid, T cell receptor (TCR)-induced interferon (IFN)-γ production and preformed perforin.
2. A $CD45RA^-$, $CCR7^+$ subset, which was considered to represent antigen-experienced cells because they lacked the naive CD45 isoform, but, interestingly, resembled naive cells in lacking potential effector functions. The expression of CCR7 would enable these cells to respond to CCL21 in secondary lymphoid organs. Hence, these cells were termed ''central memory'' T cells (T_{CM}).
3. A $CD45RA^-$, $CCR7^-$ subset that was considered also to include memory cells, but which differed from the T_{CM} subset not only by lacking expression of CCR7 but also in having a capacity for rapid, TCR-induced IFN-γ and preformed cytoplasmic granules containing perforin. The absence of CCR7 and expression of CCR5 implied impaired homing to secondary lymphoid organs and, instead, more likely localization to peripheral tissue sites where effector function would be appropriate. Therefore, this population was termed the effector memory (T_{EM}) subset.
4. A fourth subset of $CD45RA^+$, $CCR7^-$ cells that also were capable of rapid production of IFN-γ and stained even more intensely for perforin. These were termed effector cells (T_{EFF}) that had reverted to the $CD45RA^+$ isoform of naive cells, which is a characteristic of highly differentiated, antigen-experienced cells (Michie *et al.*, 1992).

This report also indicated that *in vitro* stimulation of $CD4^+$ T_{CM} caused these cells to acquire a T_{EM} phenotype, but that stimulation of $CD4^+$ T_{EM}

did not induce a T_{CM} phenotype. *In vitro* stimulation of naive CD4$^+$ T cells generated cells with a T_{CM} phenotype. These findings suggested a pathway of antigen-dependent development that was, naïve → T_{CM} → T_{EM}, proceeding from least to most differentiated with respect to effector function. However, the relevance of *in vitro* to *in vivo* differentiation is unclear, and this analysis was performed with CD4$^+$ T cells rather than CD8$^+$ T cells.

Finding that homing potential correlated with effector function suggested that antigen-dependent development of CD8$^+$ T cells was regulated rather than stochastic, and by defining distinct subsets of antigen-experienced cells (Sallusto *et al.*, 1999) implicitly raised important questions of what the functions of the subsets are, and how these subsets develop during cellular responses to acute and persistent infections. Left unresolved by this study was the question of whether these subsets of cells identified only by CD45 isoforms and CCR7 were themselves developmentally homogenous or even could be further subdivided, and whether analysis of resting cells in peripheral blood could be extended, without modification, to actively proliferating cells in lymphoid and extralymphoid tissue. Nevertheless, in many ways this study has set the agenda for research in the development of antigen-experienced CD8$^+$ T cells, which is remarkable in that it was a noninterventional study of human lymphocytes rather than an interventional study of the murine CD8$^+$ T cell system.

1.3. Central memory CD8$^+$ T cells generate new effector CD8$^+$ T cells

To establish the relevance of Sallusto *et al.* (1999) to the problem of how CD8$^+$ T cells generate new T_{EFF} in persistent or recurrent infections, subsets of murine memory CD8$^+$ T cell subsets corresponding to the human subsets needed to be identified and assessed for differences in their replicative function when challenged by viral infection *in vivo*. In 2003, Ahmed and his colleagues (Wherry *et al.*, 2003) reported that in mice that had been infected with *Lymphocytic choriomeningitis virus* (LCMV) 2–3 months previously, two populations of memory CD8$^+$ T cells could be distinguished based on their relative expression of the homing receptor, CD62L. The CD62Lhigh cells were CCR7$^+$ and the CD62Llow cells were CCR7$^-$, so these two murine memory CD8$^+$ T cell populations appeared to correspond to the human T_{CM} and T_{EM} subsets, a correlation that seemed reasonable since both CCR7 and CD62L promote migration of cells from blood into secondary lymphoid organs. However, the correspondence was not exact because the murine T_{CM} and T_{EM} both were capable of rapid production of IFN-γ while only human T_{EM} but not T_{CM} had this function. Despite these differences, especially with respect to

an important marker of effector differentiation, the CD62Lhigh memory $CD8^+$ T cell was considered to be equivalent to the human T_{CM} and to be developmentally distinct from the CD62Llow memory $CD8^+$ T cell, which were termed T_{EM}, even though both had the effector function of potential IFN-γ production.

Nevertheless, the distinction of LCMV-specific memory subsets by relative CD62L expression was useful because after adoptive transfer and challenge of recipient mice with relevant viruses, the two subsets were functionally distinct. T_{CM} provided more effective immunity to the viral challenge than T_{EM} and demonstrated greater clonal expansion and generation of new T_{EFF}, even though only a modest enhancement in replicative function relative to T_{EM} was observed *in vitro*. This observation that CD62Lhigh memory $CD8^+$ T_{CM} were more effective in secondary clonal expansion than CD62Llow T_{EM} has been confirmed in almost all studies using a similar protocol of assessing adoptively transferred memory cells obtained from mice that had resolved acute, primary viral infections (Bachmann *et al.*, 2005; Bouneaud *et al.*, 2005; Marzo *et al.*, 2005). Perhaps even more importantly for the intent of this chapter, this functional distinction even holds for $CD8^+$ T cells specific for the ''inflationary'' immediate-early 1 (IE1) epitope taken from mice with persistent infection with murine cytomegalovirus (mCMV) (Pahl-Seibert *et al.*, 2005) (see Section 2). Therefore, T_{CM} are responsible for generating new T_{EFF}, whether taken from mice during antigen-free ''memory'' periods or during persistent viral infections in which there has been continual antigenic stimulation.

1.4. Current models for development of antigen-stimulated $CD8^+$ T cells

With the discovery of heterogeneity in memory $CD8^+$ T cells that, in humans at least, was reflected by cells differing in their extent of effector differentiation and in the mouse by cells with differing *in vivo* proliferative potential, two general proposals have been advanced to account for the development of antigen-stimulated $CD8^+$ T cells. The first was suggested by analogy with the developmental pathways of other organ systems, and postulated that, ''A stem cell-like capacity for self-renewal could be the basis for the continual generation of effector lymphocytes from the memory pool'' (Fearon *et al.*, 2001). This stem cell stage would provide the pool of replicating precursor cells from which could emerge $CD8^+$ T cells that commit to effector differentiation. These differentiating cells would have limited replicative potential, but asymmetrical division in the stem cell pool would insure maintenance of undifferentiated cells with relatively unlimited replicative potential. Since the cytokine, IL-2, was known to drive effector differentiation, clonal expansion mediated by

IL-2 was considered to be responsible for generating the T_{EM} and T_{EFF} subsets of antigen-experienced $CD8^+$ T cells. Therefore, the existence of an IL-2-independent pathway for $CD8^+$ T cell clonal expansion was proposed for the development of the self-renewing, nondifferentiating pool of cells that would maintain replicative function (Fearon *et al.*, 2006). Being undifferentiated, the self-renewing cells would be contained within the T_{CM} subset, so the proposal was consistent with the apparent precursor relationship of human $CD4^+$ T_{CM} to $T_{EM/EFF}$ (Sallusto *et al.*, 1999).

This stem cell model has been supported by recent findings of an IL-2-independent pathway of clonal expansion for the $CD8^+$ T cell that generates cells having a T_{CM} phenotype and lacking effector functions (Carr *et al.*, 2006), which will be discussed in more detail in Section 4 , and by the demonstration of asymmetrical division of $CD8^+$ T cells *in vivo* (Chang *et al.*, 2007). Asymmetrical division, which is central to the concept of a self-renewing cell, was supported in this study by the finding that at the first cellular division of $CD8^+$ T cells in response to microbial antigenic challenge, one daughter cell contained granzyme B, was capable of IFN-γ production, exhibited short-term host defense, but had diminished long-term protective capability, while the other daughter cell lacked immediate effector functions but had better long-term antimicrobial activity, possibly reflecting better replicative function, although this was not shown.

The second, and perhaps more generally accepted model is termed ''linear differentiation,'' and was presented as a consequence of finding that memory $CD8^+$ T cells with a T_{CM} phenotype had better secondary proliferative function than did T_{EM} cells and that T_{CM} apparently could not be detected during acute, primary $CD8^+$ T cell clonal expansion (Wherry *et al.*, 2003). Adoptive transfer to naive recipients of LCMV-specific $CD8^+$ T cells taken from mice at various times during and after acute LCMV infection followed by viral challenge showed that $CD8^+$ T cells with secondary proliferative function were not detectable at the peak of the acute primary response, when many T_{EFF} were present, but gradually appeared during the weeks following resolution of the primary infection. As this coincided with a change in phenotype of the LCMV-specific $CD8^+$ T cells from $T_{EFF/EM}$ (CD62Llow) to T_{CM} (CD62Lhigh), the authors concluded, ''Thus, the findings of our study and the proposed model of linear differentiation (Naïve → Effector → T_{EM} → T_{CM}) are likely to provide the paradigm for acute infections. We propose that this will be the natural course of memory T cell differentiation in the absence of antigen. It is possible, however, that under certain conditions, especially chronic infections where antigen persists at high amounts, one may see a different pattern of memory T cell differentiation.'' The latter comment refers to the phenomenon of ''exhaustion'' of $CD8^+$ T cells in mice infected with clone 13 LCMV. High levels of virus persist beyond the

acute phase of infection, and antigen-specific $CD8^+$ T cells demonstrate impaired TCR signaling and an inability to replicate, perhaps because of the expression of the inhibitory receptor PD-1 (Barber *et al.*, 2006) or excessive IL-10 production (Brooks *et al.*, 2006). However, these interesting observations may not be relevant to the response of the $CD8^+$ T cell system to other persistent viral infections in which $CD8^+$ T cells do maintain an ability to generate new T_{EFF}, as will be discussed in Section 2.

Perhaps the most controversial aspect of the linear differentiation pathway is its requirement that antigen-experienced $CD8^+$ T cells be capable of dedifferentiation in the absence of antigenic stimulation, with a gradual loss of effector function and regaining of a level of proliferative function that is at least equivalent to that of the naive $CD8^+$ T cell. Although the findings in this study (Wherry *et al.*, 2003) seemed to indicate that dedifferentiation occurred, and the complexity of dealing with diverse and changing microbial targets has often selected for unique biological capabilities in the adaptive immune system, such a capability would be unusual in a general developmental biology context. Furthermore, a subsequent study reported that cells that had converted their CD62L phenotype during the memory phase did not have replicative function (Bouneaud *et al.*, 2005), another found that antigen-specific CD62Lhigh, $CD8^+$ T cells with high proliferative function could be found during the peak of the primary response (Bachmann *et al.*, 2005), and a third concluded that a capacity for conversion from T_{EM} to T_{CM} may reflect incomplete differentiation (Marzo *et al.*, 2005). Moreover, in addition to differentiation-associated changes in transcription of the CD62L gene, low expression of CD62L may be induced by TCR-induced, metalloproteinase-mediated cleavage of the ectodomain of CD62L, which is reversible if further changes in the developmental status of the cell have not occurred (Chao *et al.*, 1997; Jung *et al.*, 1988). Finally, cells with a T_{CM} phenotype survive better than do those with T_{EM} and T_{EFF} phenotypes, perhaps because they maintain expression of CD127, the IL-7Rα chain (Kaech *et al.*, 2003; Schluns *et al.*, 2000), and the gradual increase in the T_{CM}/T_{EM} ratio during the memory phase could also have been caused by the homeostatic expansion of T_{CM} in response to IL-15 (Becker *et al.*, 2002; Goldrath *et al.*, 2002; Tan *et al.*, 2002). Therefore, although dedifferentiation might occur with a ''transitional'' type of antigen-stimulated $CD8^+$ T cell, perhaps accounting for the secondary replicative function of memory $CD8^+$ T cells that had received IL-2R signals during the primary response (Williams *et al.*, 2006), the essential prediction of the linear differentiation model is that the first step in development of the antigen-stimulated $CD8^+$ T cell is differentiation to T_{EFF}, and that cells with long-term replicative function are derived from these T_{EFF}.

This prediction of the linear differentiation pathway implies that whenever an acute infection is not cleared, the $CD8^+$ T cell system will

necessarily fail. There would be an inability of antigen-specific CD8$^+$ T cells to maintain the production of new T_{EFF} because there would be no antigen-free ''rest period'' to allow dedifferentiation of T_{EFF} to T_{CM} with acquisition of replicative function. Although this circumstance is compatible with the CD8$^+$ T cell response to an acute viral infection, such as LCMV, it cannot explain the success of the CD8$^+$ T cell system in controlling persistent viral infections, such as those caused by the herpes viruses. However, as these viruses cease replicating after the acute phase and enter into the latent phase of infection, one could suggest that latency is equivalent to an antigen-free period. Section 2 will discuss whether antigenic stimulation ever fully ceases during the latent phase of herpes virus infection, at least for all antigens, but even more persuasive evidence for the ability of the CD8$^+$ T cell system to maintain a continuous production of T_{EFF} during long-term, active viral infections is its ability to control infection with human immunodeficiency virus (HIV) until the CD4$^+$ T cell response is lost. The occurrence of HIV escape mutants must indicate the continued generation of functional T_{EFF} to account for the selection of the mutants. Therefore, better insight into the developmental program of antigen-experienced CD8$^+$ T cells may be gained by an analysis of persistent rather than acute viral infections.

2. THE BEHAVIOR OF THE CD8$^+$ T CELL IN PERSISTENT VIRAL INFECTIONS

This section will review the evidence that persistent viral infections cause continuous antigenic stimulation of the CD8$^+$ T cell, that cellular replicative senescence occurs with highly differentiated CD8$^+$ T cells, that despite the occurrence of senescence, an antigen-specific CD8$^+$ T cell response is maintained that reflects clonal maintenance rather than clonal succession, all of which strongly suggests the existence of a self-renewing stage of antigen-dependent development.

2.1. Persistent CD8$^+$ T cell stimulation and expansion: "Inflationary" epitopes

Reddehase and colleagues have proposed a ''silencing/desilencing and immune sensing'' hypothesis by which CD8$^+$ T cells control CMV latency by epitope-specific sensing of transcriptional reactivation of the virus. CD8$^+$ T cells specific for an IE1 epitope recognize and terminate virus reactivation *in vivo* at the first opportunity in the reactivated gene expression program (Reddehase *et al.*, 1989; Simon *et al.*, 2006). This CD8$^+$ T cell response is caused by viral reactivation rather than being a unique attribute of the IE1 peptide epitope because similar CD8$^+$ T cell responses

occur with other epitopes when their expression is regulated by the IE1 promoter in recombinant viruses (Karrer *et al.*, 2004). The continual presentation of the IE1 epitope in a small proportion of latently infected cells causes an ''inflation,'' or continuous expansion, over time of $CD8^+$ T cells specific for this epitope (Karrer *et al.*, 2003). Most $CD8^+$ T cells having an inflationary response had a highly differentiated phenotype of CD28low, CD27low, CD122low, and CD62Llow, whereas $CD8^+$ T cells specific for epitopes that induced expansion during the acute phase but not during the latent phase of mCMV infection showed ''a slow reversion'' to the T_{CM} phenotype (Sierro *et al.*, 2005), as had been observed with memory $CD8^+$ T cells specific for LCMV (Wherry *et al.*, 2003). From this and other similar observations (Holtappels *et al.*, 2000; Munks *et al.*, 2006), it was concluded that a particular memory phenotype is determined by the frequency of TCR stimulation, with continually presented epitopes causing a $T_{EM/EFF}$ phenotype, and epitopes that are not presented during latency being associated with a T_{CM} phenotype. The prediction of the linear differentiation model would be that clones composed of $CD8^+$ T cells with highly differentiated $T_{EM/EFF}$ phenotype would no longer generate new effector cells, but since the continued $CD8^+$ T cell response to the IE1 epitope is necessary to control reactivation of mCMV (Simon *et al.*, 2006), it is likely that there is continued generation of new T_{EFF}, although cellular turnover studies are needed to confirm this conclusion. Either $CD8^+$ T cells with a $T_{EFF/EM}$ phenotype can replicate in mCMV-infected mice, in contrast to the response of T_{EFF} from LCMV-infected mice (Wherry *et al.*, 2003), or there is a source in mCMV-infected mice of IE1-specific $CD8^+$ T cells having the T_{CM} phenotypic characteristic of replicative competence. The latter possibility was experimentally supported when a population of CD62Lhigh, IE1-specific $CD8^+$ T cells in the lymph nodes of infected mice was found to have remarkable proliferative function after adoptive transfer to naive mice and challenge with mCMV (Pahl-Seibert *et al.*, 2005).

Although mCMV is perhaps the best experimental system for addressing the question of how the $CD8^+$ T cell responds to continual stimulation, analyses of other murine herpes viruses are consistent with the conclusions drawn from the mCMV studies. For example, latent infection with herpes simplex virus (HSV) infection is also associated with continual $CD8^+$ T cell stimulation (Khanna *et al.*, 2003; van Lint *et al.*, 2005), and these $CD8^+$ T cells may be required to prevent reactivation in sensory ganglions (Liu *et al.*, 2000). Also, HSV-specific $CD8^+$ T cells from latently infected mice having a CD62Lhigh, T_{CM} phenotype proliferated as well as naive cells after adoptive transfer and viral challenge while cells with the same specificity but a T_{EFF} phenotype did not (Stock *et al.*, 2006). Therefore, the $CD8^+$ T cell system copes with persistent antigenic stimulation and maintains a capacity for generating new T_{EFF} apparently by

maintaining a pool of less differentiated cells within the phenotypic T_{CM} subset.

2.2. Cellular senescence despite continued clonal expansion

The studies with mCMV and HSV suggest that the continued production of T_{EFF} is mediated by replication of less differentiated $CD8^+$ T cells, but additional evidence for replicative senescence in T_{EFF} is necessary to exclude the possibility that T_{EFF} numbers are sustained by replication of these cells. Indeed, a reasonable objection to a proposal for a self-renewing, less differentiated subset of antigen-experienced $CD8^+$ T cells has been based on the well-established ability of immunologists to maintain clones of murine $CD8^+$ T cells *in vitro* by periodic restimulation with antigen and IL-2. However, the occurrence of replicative senescence has been demonstrated in several circumstances.

Senescence was induced *in vivo* by repetitive cycles of adoptive transfer of LCMV-specific TCR transgenic $CD8^+$ T cells and infection with LCMV. After each cycle of infection, a higher proportion of the $CD8^+$ T cells expressed the killer cell lectin-like receptor G1 (KLRG1), and the expansion of the restimulated $CD8^+$ T cells correspondingly diminished (Voehringer *et al.*, 2001). The $KLRG1^+$ $CD8^+$ T cells also demonstrated diminished proliferation *in vitro* in response to antigenic stimulation. It is not obvious why in these experiments a self-renewing subset was not maintained, but this may have been caused by the use of splenocytes for recovery of the antigen-experienced, LCMV-specific $CD8^+$ T cells rather than lymph node cells, which may select more effectively for CD62L high cells.

The association between expression of KLRG1 and persistent antigenic stimulation has also been demonstrated for human $CD8^+$ T cells specific for CMV, Epstein-Barr virus (EBV), and HIV (Thimme *et al.*, 2005). The $KLRG1^+$ human $CD8^+$ T cells replicated poorly in response to stimulation with phytohemagglutinin and IL-2 (Voehringer *et al.*, 2002). Similar findings of replicative senescence in association with the expression of CD57 on antigen-experienced human $CD8^+$ T cells have been reported (Brenchley *et al.*, 2003) and are possibly an important consequence of depletion of $CD4^+$ T cells in HIV-infected patients (Papagno *et al.*, 2004). $CD8^+$ T cells from ''nonprogressor'' patients maintain *in vitro* replicative function while $CD8^+$ T cells from ''progressor'' patients do not (Migueles *et al.*, 2002). Of course, it is not possible to determine from these studies of $CD8^+$ T cells from HIV patients whether the replication-incompetent state contributed to loss of control of HIV replication, or whether uncontrolled replication caused senescence of the HIV-specific $CD8^+$ T cells. However, in persistent viral infections that are controlled, senescent, antigen-experienced $CD8^+$ T cells can be identified, so that their presence does

not necessarily indicate overwhelming viral infection. Rather, it may be a normal developmental outcome of continued antigenic stimulation, as it appears to be in mice and humans with CMV infections.

2.3. Clonal persistence versus clonal succession

Two general processes could maintain the long-term generation of $CD8^+$ T_{EFF} in persistent viral infections: the maintenance of clones that are selected by antigen early in the antiviral response or the replacement of depleted clones by recruitment of naive $CD8^+$ T cells, a process that is termed clonal succession. A recent report has suggested that clonal succession contributes to the murine response to persistent infection with polyomavirus, although a requirement for clonal succession was not demonstrated (Vezys *et al.*, 2006). The relevance of this finding to the response of human $CD8^+$ T cells to CMV (Khan *et al.*, 2002; Weekes *et al.*, 1999), EBV, and HIV (Cohen *et al.*, 2002) is unclear as long-term persistence of virus-specific clones was demonstrated in each of these analyses. Given these studies tracking $CD8^+$ T cell clones by the use of CDR3-specific probes, and the continued control of persistent viral infections in aging adults experiencing normal thymic involution, it seems likely that the essential means for maintaining an antiviral response in persistent infections is clonal maintenance rather than clonal succession.

2.4. Molecular requirements for clonal persistence

Relative to the many studies of different genetically modified mice in classical memory protocols, there are relatively few reports of the signaling pathways that mediate the maintenance of repetitively stimulated $CD8^+$ T cells in persistent viral infections. In mice infected with γ-herpesvirus or HSV there is a requirement for $CD4^+$ T cells (Cardin *et al.*, 1996), at least in part for their role in ''licensing'' of dendritic cells (Smith *et al.*, 2004) by stimulation through CD40 (Sarawar *et al.*, 2001), and for CD27 on either $CD4^+$ or $CD8^+$ T cells (Kemball *et al.*, 2006). The need for $CD4^+$ T cell-dependent activation of dendritic cells through CD40L–CD40 interaction had been previously recognized in the generation of some primary (Bennett *et al.*, 1998; Ridge *et al.*, 1998; Schoenberger *et al.*, 1998) and memory $CD8^+$ T cell responses (Janssen *et al.*, 2003; Shedlock and Shen, 2003; Sun and Bevan, 2003), which may be related to inducing the expression on dendritic cells of CD70, the ligand for CD27. The possible central roles of CD70- and CD27-mediated responses in the IL-2-independent clonal expansion of the $CD8^+$ T cell are discussed in more detail in Section 4. The potential clinical relevance of a role for $CD4^+$ T cells in persistent viral infections is, of course, the loss of control by $CD8^+$ T cells

of viral replication in CD4$^+$ T cell-deficient patients with acquired immune deficiency syndrome secondary to HIV infection.

There is an interesting possible contrasting requirement for IL-15 in the responses of memory and persistently stimulated CD8$^+$ T cells. While this cytokine is needed to maintain normal numbers of antigen-experienced CD8$^+$ T cells during the memory phase between infections (Becker *et al.*, 2002; Goldrath *et al.*, 2002; Tan *et al.*, 2002), it may not be necessary to maintain the generation of sufficient T_{EFF} for control of persistent γ-herpesvirus or HSV infections (Obar *et al.*, 2004; Sheridan *et al.*, 2006). This finding emphasizes the need to identify the antigen-experienced CD8$^+$ T cell pool that is IL-15 dependent. Although it might be argued that persistent antigenic stimulation obviates the need for sustaining antigen-experienced CD8$^+$ T cells during antigen-free period of a classical memory response, an alternative explanation may be that cells contained within the T_{CM} pool, which mediate the continual generation of new T_{EFF} (Stock *et al.*, 2006), do not require IL-15. This possibility would be consistent with the finding of a quantitatively normal secondary response of memory CD8$^+$ T cells to LCMV in IL-15-deficient mice (Becker *et al.*, 2002). This issue has implications for determining the transcription factors that are necessary to establish the self-renewing subset of antigen-experienced CD8$^+$ T cells. T-bet and eomesodermin (eomes) have been considered to be required for memory CD8$^+$ T cell maintenance based on their role in increasing expression of CD122, the IL-2βR (Intlekofer *et al.*, 2005) that mediates signaling by IL-15. If the cells in the T_{CM} subset that maintain the continuous production of new T_{EFF} do not need IL-15 signaling, then eomes and T-bet may not necessarily be involved in the development of this important population in for either classical memory or persistent viral responses.

3. CLARIFYING THE ROLE OF IL-2 IN THE CLONAL EXPANSION AND EFFECTOR DIFFERENTIATION OF THE CD8$^+$ T CELL

3.1. Is IL-2 "the" or "a" mediator of CD8$^+$ T cell clonal expansion?

IL-2 has been considered to be the principle mediator of the clonal expansion of T cells since its initial identification as the ''T cell growth factor'' 20 years ago (Cantrell and Smith, 1984; Gillis and Smith, 1977; Morgan *et al.*, 1976). Ligation of the TCR induces expression of CD25, the IL-2Rα chain, that enables high-affinity binding of IL-2 to the IL-2R and transcription of the IL-2 gene, perhaps with the assistance of signals from CD28. The apparent simplicity of this system and its ability to maintain the long-term

growth *in vitro* of murine $CD8^+$ T cell clones supported its candidacy as the mediator of the clonal expansion of the antigen-stimulated T cell, a obligatory cellular response in the clonal selection principle of adaptive immunity. More recent reports showed that even if the $CD8^+$ T cell did not itself provide IL-2 for autocrine stimulation, relatively transient ligation of the TCR ''programmed'' the cell for a paracrine IL-2 response that extended for 7–10 cell cycles (Kaech and Ahmed, 2001; Wong and Pamer, 2001). Observations such as these seemed to confirm suggestions made for many years that $CD4^+$ T cell ''help'' for $CD8^+$ T cell responses was mediated by paracrine IL-2, although the role of $CD4^+$ T cells in $CD8^+$ T cell responses had been shown to be via the activation of dendritic cells (Bennett *et al.*, 1998; Ridge *et al.*, 1998; Schoenberger *et al.*, 1998). It is interesting to note that an emphasis on the role of IL-2 in clonal expansion leads logically to the linear differentiation pathway in which T_{EFF} are the generated directly from naive cells because IL-2-induced $CD8^+$ T cell replication is coupled to effector differentiation, with the assistance of additional cytokine signals (Mescher *et al.*, 2006).

Other findings suggest that prior IL-2R signaling may have negative or positive effects on subsequent antigen-dependent $CD8^+$ T cell proliferation, with reasons for the differing outcomes not being evident. For example, IL-2 was shown to program T cells for cell death in a process that has been termed, antigen- or activation-induced cell death (AICD), such that repetitive ligation of the TCR on T cells that had been stimulated by IL-2 caused an apoptotic response (Lenardo, 1991). In contrast, memory $CD8^+$ T cells that had received IL-2R signals during a primary response expanded better on rechallenge than those that had not, when assessed in the same mouse (Williams *et al.*, 2006). This finding has not been reconciled with the prior demonstration of normal secondary expansion of memory $CD8^+$ T cells when all memory cells lacked prior IL-2R signals (Yu *et al.*, 2003). Of course, even being able to evaluate memory $CD8^+$ T cells that have not received IL-2R signals indicates that IL-2 is not required for primary clonal expansion or for the development of T_{CM}. This outcome would not have been predicted by the linear differentiation model in which T_{CM} develop from T_{EFF} via T_{EM}, since in the absence of IL-2, the development of T_{EFF} does not occur (Yu *et al.*, 2003), and, of even greater significance, it is not consistent with IL-2 being the essential driver of clonal expansion.

3.2. $CD8^+$ T cell clonal expansion without IL-2R signaling

In F5 TCR-transgenic, IL-2-deficient mice, administration of antigenic peptide induced the expansion of the transgenic $CD8^+$ T cells but did not cause them to develop CTL activity (Kramer *et al.*, 1994). Thus, more than 10 years ago, immunologists were confronted with the possibility

that in the absence of IL-2, clonal expansion without effector differentiation occurs in the antigenically stimulated $CD8^+$ T cell. The nonredundant role of IL-2 in effector differentiation, in contrast to its apparently nonessential role in proliferation in this study, was also demonstrated by the development of CTLs when IL-2 was coadministered with antigenic peptide. Perhaps this study was not considered to be definitive because it did not involve a ''physiological'' stimulus for the activation of $CD8^+$ T cells, a microbial infection, but in retrospect, it is of great interest.

Other investigators did evaluate the $CD8^+$ T cell response in IL-2-deficient mice with more ambiguous outcomes, which may have been related to the autoimmunity that is caused by the absence of IL-2-dependent regulatory T cells and to difficulties associated with assessing clonal expansion before pMHC tetramers were available, which required measuring antigen-specific $CD8^+$ T cells based on their effector function, an obvious problem in IL-2-deficient conditions where effector differentiation may not occur. However, the question was addressed more recently by two groups who overcame these problems either by introducing the deficiency of IL-2Rα chain into a TCR-transgenic, Rag-deficient background (D'Souza and Lefrancois, 2003), or by restricting the deficiency of the IL-2Rβ chain to postthymic cells, thereby permitting development of the IL-2-dependent regulatory T cells (Yu *et al.*, 2003). Viral stimulation of cells unable to respond to IL-2, or even to IL-15 in the IL-2R$\beta^{-/-}$ mice, caused normal primary clonal expansion in secondary lymphoid organs. Interestingly, expansion of the IL-2R$\alpha^{-/-}$ $CD8^+$ T cells was impaired in peripheral, nonlymphoid tissues in which differentiated T_{EFF} would be expected to accumulate, suggesting a two-step process of clonal expansion with IL-2 being required only for a later phase that is associated with the accumulation of effector cells in peripheral tissues. This possibility was directly demonstrated by the absence of effector functions in *ex vivo* assays in the expanded IL-2R$\beta^{-/-}$, virus-specific $CD8^+$ T cells, demonstrating again the nonredundant role of IL-2 in T_{EFF} generation. Furthermore, the memory cells that developed in the IL-2R$\beta^{-/-}$ mice were capable of a quantitatively normal response to secondary viral infection, which suggests a developmental pathway for T_{CM} that does not involve prior differentiation to T_{EFF}.

Therefore, IL-2R signaling is not required for clonal expansion of the $CD8^+$ T cell or for the generation of the subset of memory cells that mediates secondary expansion, but is required for the development of T_{EFF}. These findings would fit easily with the model of antigen-dependent $CD8^+$ T cell development that proposes the occurrence of asymmetrical division of undifferentiated, self-renewing cells, as it presents the possibility of two pathways for clonal expansion, an IL-2-independent pathway that does not cause effector differentiation, enabling expansion through a process of self-renewal, and an IL-2-dependent pathway that

is coupled to differentiation. Although these findings of IL-2-independent clonal expansion do not exclude a linear differentiation pathway, they imply a means for maintaining persistently stimulated $CD8^+$ T cell clones because avoiding IL-2-dependent effector differentiation during clonal expansion provides a means for evading replicative senescence and AICD.

4. CORECEPTORS MEDIATING IL-2-INDEPENDENT $CD8^+$ T CELL CLONAL EXPANSION

Knowing that quantitatively normal primary clonal expansion of $CD8^+$ T cells occurs without IL-2R signaling allows one to infer that if abnormal expansion is observed when signaling through coreceptor on $CD8^+$ T cells is interrupted, that coreceptor may mediate IL-2-independent $CD8^+$ T cell proliferation. CD27 is the best example of the result of such reasoning.

4.1. CD27

CD27 and its ligand, CD70, have been known to promote $CD8^+$ T cell proliferation *in vitro* for many years (Lens *et al.*, 1998), but a nonredundant role has become evident only relatively recently (Borst *et al.*, 2005). CD27 is expressed on all naive $CD8^+$ T cells and appears to be lost only when they become highly differentiated. CD70 is expressed by dendritic cells that have been activated by both innate and adaptive immune signals, as is discussed below, and also on activated B cells. An important advance occurred when CD27-deficient mice were shown to have impaired primary and secondary expansion of $CD8^+$ T cells in response to infection with influenza (Hendriks *et al.*, 2000). CD27 has also been found more recently to be necessary for the long-term $CD8^+$ T cell response to persistent polyomaviral infection (Kemball *et al.*, 2006).

The possibility that CD27 drives IL-2-independent responses of the $CD8^+$ T cell *in vivo* is supported by finding that stimulating IL-$2^{-/-}$ $CD8^+$ T cells *in vitro* with repetitive antigen and a recombinant form of soluble CD70 caused marked clonal expansion, no change in the CD62Lhigh status, and no effector differentiation (Carr *et al.*, 2006). Thus, the expanding cells more closely resembled the T_{CM} of Sallusto *et al.* (1999) than of Wherry *et al.* (2003) in that they had not acquired a capacity for rapid synthesis of IFN-γ. The effect of CD27 on cell expansion was the result of both enhanced cell cycling and survival, with the latter being dependent on the ability of ligated CD27 to maintain the expression of IL-7Rα on TCR-stimulated cells. Since IL-7Rα expression contributes to the viability of activated cells after resolution of the acute phase of clonal expansion (Schluns *et al.*, 2000), this effect of CD27 costimulation may be especially

important for long-term clonal expansion in persistent viral infections. Moreover, in contrast to IL-2R-stimulated cells (Lenardo, 1991), repetitive TCR ligation of CD27-stimulated CD8$^+$ T cells did not induce AICD or cause the loss of *in vivo* replicative function (Gattinoni *et al.*, 2005), but instead maintained the cellular response to CD70 *in vitro* and a capability for clonal expansion and effector differentiation after adoptive transfer and viral challenge *in vivo*. The additional observation that stimulation through CD27 selectively suppressed IL-2R-induced effector differentiation, while not impairing the proliferative response to IL-2 suggests that CD27 could mediate self-renewal of the CD8$^+$ T cell even in the presence of IL-2. Taken together, these two studies of Hendriks *et al.* (2000) and Carr *et al.* (2006) make CD27 a reasonable candidate for a coreceptor that drives TCR-dependent, IL-2-independent generation of the nondifferentiating, self-renewing subset of antigen-experienced CD8$^+$ T cells.

Recent findings of the role of CD70 on dendritic cells support a critical function for CD27 stimulation of the CD8$^+$ T cell. The ability of agonistic anti-CD40 antibody to promote CD8$^+$ T cell clonal expansion was inhibited by blocking antibody to CD70 (Rowley and Al-Shamkani, 2004), and the effect of agonistic anti-CD40 antibody was shown to be on the dendritic cell (Bullock and Yagita, 2005; Sanchez *et al.*, 2007; Schildknecht *et al.*, 2007; Taraban *et al.*, 2004, 2006). Thus, earlier studies of the ability of agonistic anti-CD40 antibody to replace the function of CD4$^+$ T cells in persistent γ-herpesvirus infection (Sarawar *et al.*, 2001) and of the role of CD4$^+$ T cells in CD8$^+$ T cell responses in general may be related to inducing dendritic cell-associated CD70 to maintain the IL-2-independent pool of undifferentiated, antigen-experienced CD8$^+$ T cells. This TCR/CD27 pathway of CD8$^+$ T cell clonal expansion may also mediate the effect of CD70-expressing antigen-presenting cells in the laminia propria, which contributes to mucosal immune responses to Listeria (Laouar *et al.*, 2005), and be the basis of the efficacy of blocking anti-CD70 antibody in preventing cardiac allograft rejection (Yamada *et al.*, 2005).

The study by Carr *et al.* (2006) indicates that a cell's response to CD70 requires repetitive TCR ligation, which is consistent with this being a pathway for clonal expansion in secondary lymphoid tissue where both antigen and CD70 would be available as long as a microbial infection persists and dendritic cells continue to receive TLR and CD40 signals. However, the T cell may need to receive other signals in addition to TCR and CD27 for effective clonal expansion because in mice with a transgene directing constitutive expression of CD70 on B cells, excessive T cell activation leads eventually both to B and, paradoxically, T cell depletion (Arens *et al.*, 2001; Tesselaar *et al.*, 2003). These studies did not examine the effect of the CD70 transgene in the context of a transgenic TCR that responds poorly to environmental antigens, so that the role of inappropriately

''weak'' TCR signaling, such as that which drives homeostatic expansion, was not evaluated. The unusual CD70-dependent immunodeficiency syndrome was at least partially explained by the subsequent finding that instead of T cell depletion that occurs in wild-type mice with the CD70 transgene, a T cell proliferative abnormality was observed in transgenic mice lacking CD95 (Arens *et al.*, 2005). This finding identifies Fas–FasL interactions as an essential control for CD27-dependent lymphocyte proliferation and prompts the question of how the presumed self-renewing, CD27-stimulated $CD8^+$ T cell responding to a microbial infection circumvents Fas-mediated apoptosis. Other costimulatory signals delivered by an appropriately activated dendritic cell, which would be absent from B cells constitutively expressing the transgenic CD70, may have a role.

4.2. Other coreceptors

If impaired clonal expansion of $CD8^+$ T cells does identify coreceptors for mediating an IL-2-independent response, then CD28 must also be considered as a candidate for this function. CD28-deficient mice have diminished clonal expansion of $CD8^+$ T cells in primary and secondary responses to influenza (Bertram *et al.*, 2002, 2004; Hendriks *et al.*, 2003, 2005), and mice lacking both CD27 and CD28 have essentially no primary or secondary $CD8^+$ T cell expansion (Hendriks *et al.*, 2003). Although CD28 is known to promote the production of IL-2 through transcriptional and posttranscriptional means, the normal proliferation of $CD8^+$ T cells in the absence of IL-2 excludes this as the basis for the impaired expansion associated with CD28 deficiency.

In some of these studies (Bertram *et al.*, 2002, 2004; Hendriks *et al.*, 2005), deficiency of 4–1BB, which like CD27 is a member of the tumor necrosis factor receptor superfamily, was found to diminish clonal expansion, but the defect was more prominent in the secondary than in the primary response. 4–1BB is not expressed on naive $CD8^+$ T cells, and the precise stage of antigen-dependent development of the $CD8^+$ T cell at which 4–1BB expression occurs is not clear. It may share with CD27 a capacity for IL-2-independent proliferation, but possibly at a later stage of development following IL-2-induced differentiation.

5. MODIFYING THE ANTIPROLIFERATIVE EFFECTS OF TYPES I AND II IFN

The $CD8^+$ T cell must proliferate rapidly in the presence of types I and II IFN produced by plasmacytoid dendritic cells, NK cells and NKT cells. Since IFNs are generally antiproliferative for all other cell types

(Balkwill and Oliver, 1977; Balkwill and Taylor-Papadimitriou, 1978; Lin *et al.*, 1986), this capability is perhaps unique. Remarkably, CD8$^+$ T cells not only overcome the antiproliferative effects of IFNs, but even respond to them with enhanced clonal expansion. Furthermore, CD8$^+$ T cells use IFN-γ for differentiation, in that signaling through the IFN-γ receptor (IFN-γR) induces the expression of T-bet (Glimcher *et al.*, 2004) and, since IL-2-stimulated CD8$^+$ T cells may acquire a capacity for producing IFN-γ, the cytokine has the potential for mediating an autocrine loop that induces terminal differentiation. For these reasons, it is important to evaluate how CD8$^+$ T cells regulate their responses to types I and II IFN.

5.1. The effects of types I and II IFN on CD8$^+$ T cells

IFN-γR signaling promotes apoptosis of antigen-stimulated CD8$^+$ T cells during the acute (Lohman and Welsh, 1998) and contraction phases of the primary response (Badovinac *et al.*, 2000, 2004). In an apparently opposite outcome, IFN-γ has also been observed to promote the expansion of CD8$^+$ T cells (Sercan *et al.*, 2006; Whitmire *et al.*, 2005). Similarly, type I IFN also can enhance clonal expansion of CD8$^+$ T cells by maintaining their viability (Marrack *et al.*, 1999) and proliferation *in vitro* (Curtsinger *et al.*, 2005) and *in vivo* (Ahonen *et al.*, 2004; Honda *et al.*, 2005). Most importantly, this effect of type I IFN is known to be on the CD8$^+$ T cell itself because the expansion of IFNAR$^{-/-}$ CD8$^+$ T cells in wild-type mice infected with LCMV is diminished 100-fold (Kolumam *et al.*, 2005; Thompson *et al.*, 2006).

The capacity of IFN-γR signaling, but possibly not IFNAR signaling (Lighvani *et al.*, 2001), to induce T-bet in the CD8$^+$ T cell may indicate that IFN-γ also has a unique role in differentiation. T-bet$^{-/-}$ CD8$^+$ T cells secrete less IFN-γ, have lower CTL activity (Sullivan *et al.*, 2003), and have impaired effector function in a model of type 1 diabetes (Juedes *et al.*, 2004). However, the precise role for T-bet in the function of CD8$^+$ T cells is unclear as its expression is not required for protective CD8$^+$ T cell immunity in all microbial infections (Way and Wilson, 2004), and some functions of T-bet may be replaced by its paralog, eomes (Intlekofer *et al.*, 2005; Pearce *et al.*, 2003). Perhaps T-bet induces a stage in effector development of the CD8$^+$ T cell that is not mediated by eomes, as suggested by nonredundant functions of T-bet in the expression of the IL-12Rβ2 chain (Afkarian *et al.*, 2002; Pearce *et al.*, 2003) and in the development of NK and NKT cells (Townsend *et al.*, 2004).

5.2. Regulating IFN-γR expression

The biological importance of controlling IFN-γR signaling is suggested by the finding that fully differentiated CD4$^+$ TH1 cells and CD8$^+$ T cells do not express IFN-γR2, the signal transducing subunit of the heterodimeric

receptor complex (Bach *et al.*, 1995; Pernis *et al.*, 1995; Tau *et al.*, 2001). If IFN-γR2 is ectopically expressed in $CD4^+$ T cells, the development of TH1 cells is impaired (Tau *et al.*, 2000); ecotopic expression of IFN-γR2 in $CD8^+$ T cells also inhibits the development of CTLs (Tau *et al.*, 2001). Therefore, the transcriptional downregulation of IFN-γR2 with its attendant suppression of IFN-γR signaling is required for normal development of effector T cells of both the $CD4^+$ and $CD8^+$ T cell lineages. This transcriptional downregulation occurs not only in $CD8^+$ T cell clones generated through *in vitro* culture, but also during their primary clonal expansion during acute Listeria infection (Haring *et al.*, 2005). Interestingly, IFN-γR2 expression in the antigen-stimulated cells returns after resolution of the infection in contrast to the apparently permanent repression of its expression in TH1 and CTL clones. Since genetic deletion of IFN-γR1 expression enables clonally expanding $CD8^+$ T cells to avoid *ex vivo*-induced AICD (Lohman and Welsh, 1998), decreased IFN-γR2 may be a developmentally regulated response to enhance $CD8^+$ T cell expansion. However, the means by which IFN-γR signaling promotes AICD is not known, and a previous suggestion that it was through the induction of caspase-8 (Refaeli *et al.*, 2002) is not supported by the occurrence of AICD in caspase-8-deficient T cells (Salmena *et al.*, 2003). Since AICD in $CD8^+$ T cells requires B lymphocyte-induced maturation protein-1 (Blimp-1) (Kallies *et al.*, 2006), IFN-γR signaling may cause the expression of Blimp-1 (see Section 6).

The second means for controlling IFN-γR signaling is cell biological. While IFN-γR1 resides mainly at the plasma membrane, most of IFN-γR2 is in an intracellular compartment that has not been fully characterized, with only a few hundred copies of IFN-γR2 present at the cell surface (Rigamonti *et al.*, 2000). A dipeptide motif in the cytoplasmic domain of IFN-γR2 possibly regulates trafficking to the plasma membrane (Rosenzweig *et al.*, 2004), raising the possibility that cellular signals could acutely increase or decrease the cell's potential for responding to IFN-γ. A third means of regulating IFN-γR signaling is the redistribution of IFN-γR1 to the immunological synapse (Maldonado *et al.*, 2004). If IFN-γ secretion induced by TCR ligation is also directed to this site, this redistribution of IFN-γR1 potentially could promote autocrine responses to the cytokine leading to T-bet expression and further differentiation of the $CD8^+$ T cell.

5.3. Stat1 as a "Switch" determining the effects of types I and II IFN on proliferation

The anti- and pro-proliferative effects of IFNs on $CD8^+$ T cells and other cell types suggest that a ''switch'' exists that determines which of these two opposing effects of the IFNs will occur. Such a switch was identified

10 years ago when the expression of Stat1 by fibroblasts was found to be required for type I and type II IFN to suppress serum-induced proliferation (Bromberg *et al.*, 1996). Also remarkable was the finding that in Stat1-sufficient fibroblasts, IFN-γ suppressed the induction of c-Myc by platelet-derived growth factor (PDGF), while in Stat1-deficient cells IFN-γ no longer inhibited this growth factor response, and actually transiently induced c-Myc (Ramana *et al.*, 2000). A gene profiling study showed that in Stat1$^{-/-}$ fibroblasts, IFN-γ and PDGF induced many of the same genes (Ramana *et al.*, 2001), which may help explain how IFN-γ enhances the survival and proliferation of macrophage-colony stimulating factor-stimulated Stat1$^{-/-}$ bone marrow-derived macrophages while suppressing these responses in Stat1$^{+/+}$ cells (Gil *et al.*, 2001). These findings have recently been extended to T cells with the demonstration that type I IFN suppressed the proliferation of wild-type murine T cells stimulated with phorbol ester and IL-2 but enhanced the proliferation of similarly stimulated Stat1$^{-/-}$ or Stat2$^{-/-}$ T cells (Gimeno *et al.*, 2005).

These studies point to the possibility that if the CD8$^+$ T cell had a mechanism by which it could control the level of Stat1, it could control the nature of its growth response to type I and type II IFN. Regulation of Stat1 expression by the T cell has not been reported, but Stat1 in IFN-γ-stimulated fibroblasts is subject to ubiquitin- and proteosome-mediated degradation (Kim and Maniatis, 1996), which has also been shown to occur in osteopontin-treated macrophages (Gao *et al.*, 2007). A nuclear E3 ubiquitin ligase, termed SLIM, has also been found to suppress Stat1-dependent signaling (Tanaka *et al.*, 2005). In this respect, since serine phosphorylation often targets proteins for ubiquitin modification, it is interesting that ligation of either TCR or CD28 induces phosphorylation of serine 727 in Stat1 (Gamero and Larner, 2000; Lafont *et al.*, 2000). Although phosphorylation of serine 727 in the transactivation domain is necessary for the transcriptional activity of Stat1, this or other serines that are phosphorylated by the kinase(s) involved in these responses (Tenoever *et al.*, 2007) could also cause ubiquitination and trigger rapid degradation of Stat1. Thus, there may be a means by which TCR-stimulation of the CD8$^+$ T cell could induce a posttranslational Stat1 deficiency to enable IFNs to promote rather than suppress CD8$^+$ T cell expansion.

6. TRANSCRIPTIONAL CONTROL OF REPLICATIVE SENESCENCE: BMI-1, BLIMP-1, AND BCL6/BCL6b

The molecular determinants, other than telomerase, of whether the CD8$^+$ T cell maintains cell cycling capability or has a senescent phenotype have not been described. The ability of Bmi-1, a member of the

Polycomb-group complex, to prevent senescence of the hematopoietic stem cell (Park *et al.*, 2003) and its expression in splenic T cell lymphocytes (Zhang *et al.*, 2004) suggests that it may have a role in this process. Bmi-1 was discovered as a cooperating oncogene in Eμ-myc transgenic mice (Haupt *et al.*, 1991; van Lohuizen *et al.*, 1991). It maintains self-renewing hematopoietic, cerebellar, and leukemic stem cells (Lessard and Sauvagneau, 2003; Molofsky *et al.*, 2003; Park *et al.*, 2003) by suppressing transcription of the INK4b-Arf-INK4a tumor suppressor locus whose protein products regulate pRb and p53 (Jacobs *et al.*, 1999a). Bmi-$1^{-/-}$ mice have reduced T and B cells secondary to impaired early development (van der Lugt *et al.*, 1994), and the few mature T cells that are present have diminished *in vitro* proliferative function after TCR signaling.

Several findings suggest that expression of Bmi-1 is relevant to the proliferative response of the $CD8^+$ T cell. In parallel with the increase in Bmi-1 expression in B cells responding to ligated membrane immunoglobulin (Hasegawa *et al.*, 1998), TCR stimulation has been shown to increase Bmi-1 mRNA and protein levels in murine $CD8^+$ T cells (Heffner and Fearon, 2007). The increase in Bmi-1 is likely to be related to the replication by the TCR-stimulated $CD8^+$ T cell because ''knocking-down'' Bmi-1 with a lentiviral vector expressing an appropriate shRNA suppresses $CD8^+$ T cell proliferation, and ectopic expression of Bmi-1 promotes expansion of $CD8^+$ T cells both *in vitro* and *in vivo*. Thus, Bmi-1 expression may be linked to the proliferative capability of the antigen-stimulated $CD8^+$ T cell, just as it is to the self-renewing hematopoietic stem cell.

The means by which Bmi-1 is shut off to cause replicative senescence may be related to c-Myc. c-Myc can bind to the *bmi-1* promoter and drive transcription, and haploinsufficient *c-myc*$^{+/-}$ fibroblasts have reduced Bmi-1 levels and display INK4a-dependent senescence (Guney *et al.*, 2006). Also, there is defective homeostatic expansion of *c-myc*$^{+/-}$ memory $CD8^+$ T cells (Bianchi *et al.*, 2006), which may reflect impaired cycling secondary to diminished Bmi-1. However, appropriate studies have not been done to determine whether the replicative abnormalities of lymphocytes with diminished c-Myc are caused by effects on the expression of Bmi-1. [Not relevant to this discussion, but noted for completeness, is the apparently paradoxical finding that nonphysiologically high levels of c-Myc, as occurs in Eμ-myc transgenic mice, drive *Ink4a* transcription, overcoming Bmi-1 transcriptional repression and inducing apoptosis or senescence (Jacobs *et al.*, 1999b).]

The implication of these findings in the context of the development and differentiation of the antigen-stimulated $CD8^+$ T cell is that the transcriptional repressor, Blimp-1 (Turner *et al.*, 1994), also termed PRDI based on its inhibition of the transcription of IFN-β (Keller and Maniatis, 1991), represses *c-myc* transcription in terminally differentiated plasma

cells and mononuclear phagocytes (Chang *et al.*, 2000; Lin *et al.*, 1997). Therefore, Blimp-1 may indirectly repress transcription of Bmi-1 in these cells, in which Bmi-1 mRNA has been shown to be absent (Zhang *et al.*, 2004), and in senescent, terminally differentiated CD8$^+$ T cells, in which Bmi-1 expression is diminished (Heffner and Fearon, 2007). Consistent with this possibility are the findings that Blimp-1 is expressed in ''effector memory'' CD8$^+$ T cells, mediates AICD, and suppresses the expansion of pMHC- and homeostatically stimulated CD8$^+$ T cells *in vivo* and *in vitro* (Kallies *et al.*, 2006; Martins *et al.*, 2006). Although a decrease in c-Myc was not seen when Blimp-1 was induced in T cells *in vitro*, this might be explained by cells with low c-Myc levels being selected against during culture. If Blimp-1 is found to repress *c-myc* transcription in the CD8$^+$ T cell as it does in other cells, then terminal differentiation of the CD8$^+$ T cell would be mediated by a mechanism that is remarkably similar to the B cell lineage. Furthermore, a negative regulatory role for Blimp-1 in the expression of Bmi-1 would provide a direct link between terminal differentiation, Blimp-1, and INK4a-mediated replicative senescence.

These possibilities emphasize the importance of determining the signals that induce the expression of Blimp-1. There may be several pathways for this, as Blimp-1 can be induced in a transformed B cell line solely by IL-2R signaling (Reljic *et al.*, 2000), in myeloid cell lines by macrophage-colony stimulating factor (Chang *et al.*, 2000), and in myeloid and B cell lines by the unfolded protein stress response (Doody *et al.*, 2006). The coupling of effector differentiation of the CD8$^+$ T cell to stimulation by IL-2 and IFN-γ, the occurrence of AICD in T cells stimulated by these two cytokines (Lohman and Welsh, 1998; Refaeli *et al.*, 2002), and the dependence of AICD in the CD8$^+$ T cell on Blimp-1 expression (Kallies *et al.*, 2006) suggest that IL-2 and IFN-γ may mediate the induction of Blimp-1. However, as CD4$^+$ TH2 cells, which have differentiated in response to IL-4 rather than IFN-γ signaling, also can become Blimp-1$^+$ (Kallies *et al.*, 2006; Martins *et al.*, 2006), there is likely to be more than one pathway to Blimp-1 transcription in the CD8$^+$ T cell.

The ability of BCL6 to repress the expression of Blimp-1 in the B cell (Reljic *et al.*, 2000; Shaffer *et al.*, 2000) and prevent plasma cell differentiation in germinal center B cells (Dent *et al.*, 1997; Fukuda *et al.*, 1997; Ye *et al.*, 1997), when coupled with its role and that of its paralog, BCL6b, in enhancing the generation of memory CD8$^+$ T cells and promoting the magnitude of the secondary CD8$^+$ T cell response (Ichii *et al.*, 2002, 2004; Manders *et al.*, 2005) suggest that these transcriptional repressors may suppress Blimp-1-induced terminal differentiation and loss of Bmi-1 expression in the CD8$^+$ T cell. Although neither has been reported to do this, the probable role of IL-2 in contributing to Blimp-1 expression in T cells and the ability of BCL6b to suppress the proliferative response of the CD8$^+$ T cell to IL-2 (Manders *et al.*, 2005) make this at least plausible.

However, it is not possible to discuss the cellular interactions that would favor the expression of Blimp-1 versus BCL6/BCL6b because the signals that induce the expression of Blimp-1, BCL6, or BCL6b in the $CD8^+$ T cell have not been fully defined.

7. A REFINED MODEL FOR $CD8^+$ T CELL CLONAL EXPANSION: SEQUENTIAL PHASES OF CD27-DEPENDENT SELF-RENEWAL AND IL-2-DEPENDENT DIFFERENTIATION

The analysis of the $CD8^+$ T cell response to persistent viral infections, especially those caused by human and murine CMV, is informative because it reveals capabilities of the antigen-experienced $CD8^+$ T cell that are not evident in analyses of classical memory responses of this cell. Persistent antigenic stimulation of the $CD8^+$ T cell causes continual, ''inflationary'' clonal expansion (Karrer *et al.*, 2003) with most antigen-specific cells having a senescent and highly differentiated T_{EFF} phenotype (Holtappels *et al.*, 2000; Munks *et al.*, 2006; Sierro *et al.*, 2005). Adoptive transfer experiments showed that these differentiated T_{EFF} were not able to generate additional T_{EFF}, whereas antigen-experienced $CD8^+$ T cells with a less differentiated, T_{CM} phenotype and residing in secondary lymphoid organs did have this function (Pahl-Seibert *et al.*, 2005). These findings, combined with the demonstration of long-term clonal persistence of $CD8^+$ T cells specific for continually presented viral epitopes (Cohen *et al.*, 2002; Khan *et al.*, 2002; Weekes *et al.*, 1999), lead to the conclusion that persistently stimulated clones are maintained by a process of self-renewal, with asymmetrical division yielding both undifferentiating progeny and daughter cells that become committed to effector differentiation. The quantitatively normal clonal expansion without differentiation that occurs with antigen-stimulated, IL-$2R^{-/-}$ $CD8^+$ T cells (D'Souza and Lefrancois, 2003; Yu *et al.*, 2003) demonstrates that IL-2-independent expansion is robust and that IL-2 has a nonredundant role in effector differentiation. Therefore, to avoid clonal senescence, antigen-stimulated $CD8^+$ T cells must establish a self-renewing, nondifferentiating pool that is capable of IL-2-independent expansion and that avoids IL-2-induced differentiation. This pool would serve as the source of cells that have the potential of entering a phase of IL-2-dependent expansion and effector differentiation when antigenic stimulation indicates the need for additional T_{EFF}.

Two sets of findings provide evidence that an IL-2-independent phase of clonal expansion can be mediated by CD27: first, unlike IL-2R-deficient mice, CD27-deficient mice show impaired expansion of antigen-specific $CD8^+$ T cells in acute (Hendriks *et al.*, 2000) and persistent viral infections (Kemball *et al.*, 2006), and second, ligation of CD27 on repetitively TCR-stimulated $CD8^+$ T cells *in vitro* causes IL-2-independent expansion

without effector differentiation (Carr *et al.*, 2006). The expanded cells retain the potential for infection-induced expansion and differentiation *in vivo*. In addition, costimulation through CD27 suppresses differentiation caused by IL-2 *in vitro*. Although other coreceptors, such as CD28, may also have this capability, the importance of these findings with CD27 is that they establish the principle of IL-2-independent CD8$^+$ T cell clonal expansion without differentiation, that is, self-renewal. The recent demonstration of asymmetrical division of antigen-stimulated CD8$^+$ T cells (Chang *et al.*, 2007) is consistent with this view of two means for clonal expansion, one that does not initiate differentiation and the other that does. These findings do not accommodate the linear differentiation model, which envisions effector differentiation with loss, even if only temporary, of replicative function as being the first step in the development of the antigen-stimulated CD8$^+$ T cell (Wherry *et al.*, 2003).

Superimposed on this basic, underlying process are two additional themes: first, a remarkable switch in the nature of the response of the antigen-stimulated CD8$^+$ T cell to type I and possibly type II IFN from antiproliferative to proproliferative, the mechanism for which is suggested to be related to posttranslational regulation of Stat1, and second, the control of terminal differentiation and senescence. The latter may involve the expression of Blimp-1 in the IL-2-stimulated CD8$^+$ T cell (Kallies *et al.*, 2006; Martins *et al.*, 2006); Blimp-1 is not induced by repetitive TCR and CD27 signaling. Blimp-1 may indirectly suppress the expression of Bmi-1, which may be required for the CD8$^+$ T cell to prevent replicative senescence in the antigen-experienced CD8$^+$ T cell (Heffner and Fearon, 2007) as it is in the hematopoietic stem cell. Senescence may be delayed by BCL6 (Ichii *et al.*, 2002) or BCL6b (Manders *et al.*, 2005), which, by analogy to the function of BCL6 in the germinal center B cell, may suppress the induction of Blimp-1 by inhibiting transcriptional events downstream of IL-2R signaling. These views are summarized in Fig. 3.1.

8. CLINICAL EXTENSIONS OF THE TCR/CD27 PATHWAY: ADOPTIVE CD8$^+$ T CELL THERAPY

The definition of a means for expanding antigen-specific CD8$^+$ T cells *in vitro* without causing replicative senescence after adoptive transfer and *in vivo* challenge may increase the clinical utility of adoptive CD8$^+$ T cell therapy. Two general clinical situations have been examined for adoptive CD8$^+$ T cell therapy: the treatment of persistent viral diseases that occur in individuals rendered immunodeficient by HIV infection or during the course of bone marrow transplantation and in patients with cancer. Disseminated CMV infection has been successfully treated by adoptive transfer of CMV-specific CD8$^+$ T cells, with a recent example

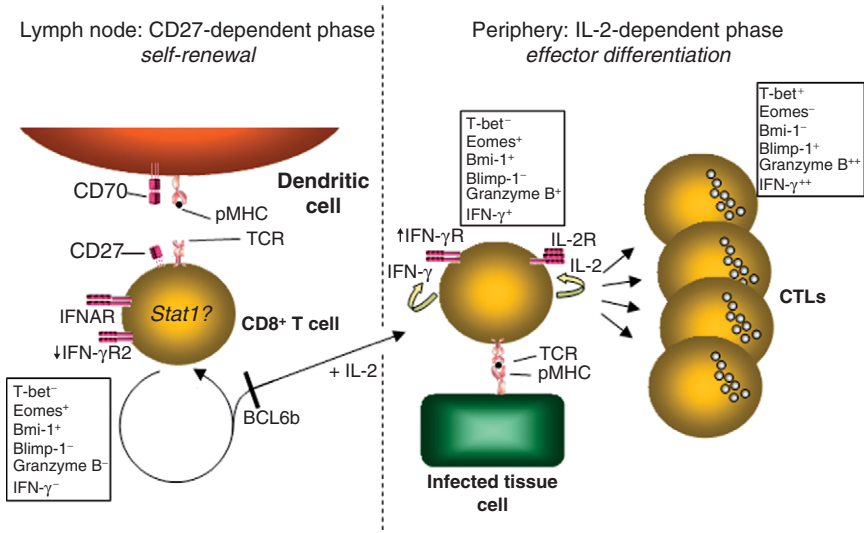

FIGURE 3.1 The two phases of central and peripheral $CD8^+$ T cell clonal expansion. (1) Dendritic cells "fully" activated by TLR ligands and CD40L present pMHC and CD70 to ligate TCR and CD27 on antigen-specific $CD8^+$ T cells. (2) TCR/CD27 signals clonal expansion. IL-2R signals may occur, but CD27 suppresses IL-2R-induced effector differentiation. (3) Repetitively ligated TCR on CD27-stimulated $CD8^+$ T cells may switch their response to type I IFN from growth inhibitory to growth enhancing, possibly through ubiquitin-mediated regulation of Stat1 transcriptional activity. (4) Unknown signals suppress IFN-γR2 expression to avoid AICD. (5) Expansion of the pool of self-renewing, antigen-specific $CD8^+$ T cells and competition for pMHC and CD70 on dendritic cells allow some T cells to initiate differentiation in response to IL-2 and eomes. These cells change their homing receptors and migrate to peripheral inflamed tissues. (6) Transcriptional repression of IL-2R signaling by BCL6 or BCL6b expands the central pool of replicating cells. (7) Encounter with pMHC in peripheral tissue causes secretion of IL-2, which maintains expansion and drives further differentiation. As autocrine and paracrine IFN-γ is produced and IFN-γR2 is reexpressed, T-bet is induced, which completes differentiation. (8) Blimp-1 levels rise as the cells differentiate, leading to suppression of Bmi-1, possibly indirectly, and cell cycle arrest.

using CMV-specific cells purified by cell sorting based on the binding of pMHC complexes bearing the relevant peptide (Cobbold *et al.*, 2005). No attempt was made to expand the cells by *in vitro* stimulation so that excessive differentiation with loss of *in vivo* replicative function did not occur. However, if expansion *in vitro* of IE1-specific $CD8^+$ T cells with maintenance of replicative function could be made possible through the TCR/CD27 pathway, a bank of CMV-specific $CD8^+$ T cells from normal individuals of differing HLA haplotypes could be established for adoptive transfer therapy. This would avoid the need to sort antigen-specific

cells acutely for immediate adoptive transfer and facilitate the use of this therapy. Such an approach could be extended to the clonal expansion of CD8$^+$ T cells specific for multiple viruses for use in immunodeficient patients who not infrequently have uncontrolled infections involving more than a single virus (Leen *et al.*, 2006). The ultimate success of this strategy would be enhanced if a similar approach could be developed for the *in vitro* expansion of CD4$^+$ T cells that will be required for the maintenance of the CD8$^+$ T cell response.

Immunological therapy of tumors has followed two general approaches (Blattman and Greenberg, 2004; Gattinoni *et al.*, 2006): first, active immunization with tumor-associated antigens in combination with other immunopotentiating agents for therapeutic treatment of patients with clinically evident tumor (Hodi *et al.*, 2003), or immunization for prophylactic treatment of patients following apparent total resection of the tumor, but in whom undetectable micrometastases may be present (Jager *et al.*, 2006); and second, adoptive T cell therapy after *in vitro* expansion of T cells specific for tumor-associated antigens (e.g., Dudley *et al.*, 2002). The advantage of therapeutic immunization is its relative simplicity, but the expansion of CD8$^+$ T cells that are specific for tumor-associated antigens may not overcome the hurdle of a local, immunosuppressive environment within the tumor itself (Willimsky and Blankenstein, 2005). Therefore, a major advantage of adoptive T cell therapy is the opportunity to alter the T cells during *in vitro* culture so that they may become resistant to immunosuppressive mediators in the tumor, such as TGF-β (Chen *et al.*, 2005; Gorelik and Flavell, 2001). However, repetitive stimulation of CD8$^+$ T cells with antigen and IL-2 ablates their antitumor effects because of loss of *in vivo* replicative function (Gattinoni *et al.*, 2005; Klebanoff *et al.*, 2005), so that genetic modification during *in vitro* clonal expansion is not feasible using the standard means for CD8$^+$ T cell proliferation. As proposed for developing antigen-specific CD8$^+$ T cells to employ in adoptive therapy for viral diseases, the use of the TCR/CD27 pathway could potentially overcome this technical problem and enhance the efficacy of this approach to cancer immunotherapy.

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