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Inflaming the CD8⁺ T Cell Response

Review

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Although inflammatory cytokines induced by infection or vaccination with adjuvants have long been known to stimulate optimal antigen-presenting cell function, recent evidence indicates that some inflammatory cytokines also act directly on the responding T cells to control their response to infection. Here, we review the evidence that specific inflammatory cytokines act to control the magnitude of expansion, the degree of contraction, and the rate of memory cell development. These data may suggest new strategies for manipulating vaccine efficacy in the quest to protect against pathogenic microbes.

Introduction

CD8⁺ T cells recognize and respond to pathogen-encoded peptides displayed by MHC class I molecules on infected cells or on antigen-presenting cells (APCs). Activated CD8⁺ T cells manifest an array of antimicrobial effector pathways and molecules, which eliminate infected cells by cytolysis or recruit and activate other immune cells (Harty et al., 2000). CD8⁺ T cells are important in defense against viruses, intracellular bacteria, and protozoan pathogens and are also potentially important in defense against malignancy. Thus, pathogen-specific CD8⁺ T cell responses are an important goal of vaccination.

The CD8⁺ T cell response can be divided into four relatively distinct phases (Figure 1). Initial activation (phase 1) is critically dependent on relatively stable interactions between naive T cells and mature, antigen (Ag) and costimulatory molecule-expressing dendritic cells (DCs) (Lanzavecchia and Sallusto, 2001). The subsequent proliferative (expansion) phase lasts 5–8 days, during which Ag-specific CD8⁺ T cell numbers may increase by >10,000-fold (Badovinac and Harty, 2002; Kaech et al., 2002b). Clonal expansion is also associated with differentiation to effector T cells that migrate throughout the body to defend against infection (Weninger et al., 2002). The third phase involves a contraction in number where 90%–95% of effector cells are eliminated over the ensuing week (Badovinac and Harty, 2002; Sprent and Tough, 2001), and the final phase is the initiation and maintenance of the memory CD8⁺ T cell pool by the cells surviving contraction (Kaech et al., 2002b). Several studies collectively indicate that the expansion, contraction, and memory phases of the CD8⁺ T cell response are largely if not completely independent of continued Ag display, suggesting that a relatively brief encounter with Ag is sufficient to instigate the full program of CD8⁺ T cell differentiation (Badovinac et al., 2002; Kaech and Ahmed, 2001; Mercado et al., 2000; van Stipdonk et al., 2001). However, it has become clear that, in addition to Ag, a variety of signals must be integrated by the responding T cells to generate optimal responses and ensure proper regulation of T cell homeostasis. Here, we discuss the evidence that inflammatory cytokines, stimulated by infection or vaccination with strong adjuvants, provide important signals that directly shape the Ag-specific CD8⁺ T cell response.

Inflammatory Cytokines Regulate T Cell Expansion

CD8⁺ T cells specific for any one peptide constitute a small fraction of the naive repertoire. This population must greatly expand in numbers to effectively combat rapidly replicating pathogens. In addition, the size of the memory CD8⁺ T cell pool, which provides protection against future exposure to the same pathogen, is directly proportional to the size of the effector T cell pool generated after primary infection or vaccination (Masopust and Ahmed, 2004). However, whereas experimental infections of mice generate robust expansion in the numbers of Ag-specific effector cells, subunit vaccines are generally less effective even when delivered with adjuvants (Levine and Sztein, 2004; Zinkernagel, 2003). Although the amount of Ag will always be an important consideration in the magnitude of the response to vaccination, these observations also suggest that the inflammatory milieu stimulated by infection contributes to robust expansion in Ag-specific CD8⁺ T cell numbers.

Proinflammatory cytokines elicited in response to infection have immediate effects on cells to promote early control of pathogen replication. Furthermore, inflammatory cytokines stimulated through microbial pattern recognition receptors (PRR) participate in generation of potent APCs to prime T cell responses. Indeed, the current paradigm is that successful vaccination depends on strong adjuvants to activate inflammation and APCs. These issues have been extensively reviewed (Castellino and Germain, 2006; Heath and Carbone, 2001; Steinman, 2003), and we will not deal further with the role of inflammation in APC biology. Instead, it has become increasing clear that T cells also receive signals from inflammatory cytokines, which determine the extent of their proliferation, survival, and ability to develop effector functions. To date, the cytokines identified that can provide this signal 3 for optimal CD8⁺ T cell expansion are interleukin-12 (IL-12), type I interferons (IFN-αβ), and type II interferon (IFN-γ).

IL-12 as Signal 3

IL-12 is produced by DCs and other cells after some, but not all, infections and is an important mediator of Th1 polarization of CD4⁺ T cells (Murphy et al., 2000). IL-12 can also act synergistically with IL-18 to induce Ag-independent IFN-γ production by memory phenotype CD8⁺ T cells (Beadling and Slifka, 2005; Berg et al., 2003; Lertmemongkolchai et al., 2001). In addition, IL-12 can act directly on CD8⁺ T cells in vitro and in vivo to promote

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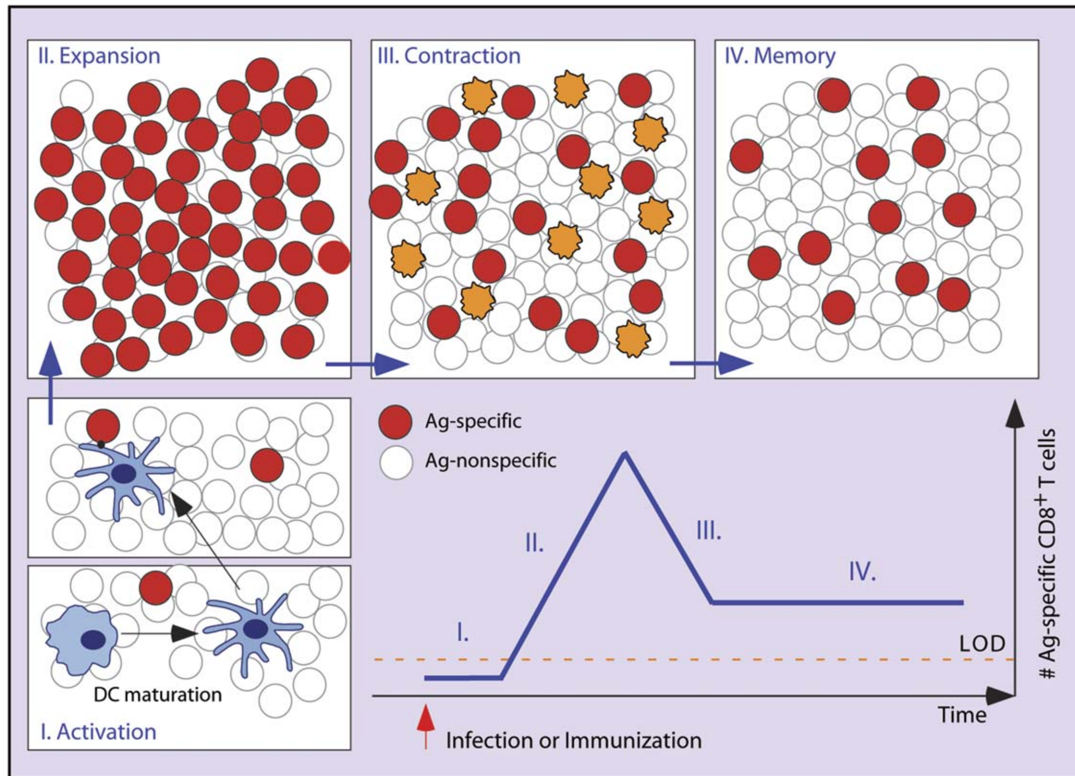

Figure 1. Naive-to-Memory CD8+ T Cell Progression after Acute Infection or Vaccination

Antigenic peptides presented by mature dendritic cells (DCs) to naive CD8+ T cells trigger expansion and differentiation into effector CD8+ T cells. Five to ten percent of CD8+ T cells detected at the peak of expansion survive the contraction phase to initiate the memory pool, which can remain stable in number for life. LOD represents limit of detection.

proliferation, survival, and differentiation into effector cells capable of cytolysis (Curtsinger et al., 1999; Schmidt and Mescher, 1999). When CD8+ T cells are exposed to IL-12 together with peptide-MHC and signal 2 (provided by either CD28-B7 interactions or IL-2), their ability to proliferate, survive, and kill is markedly enhanced compared to T cells in the absence of IL-12 (Curtsinger et al., 1999; Schmidt and Mescher, 1999, 2002).

The timing of exposure to IL-12 is critical for optimal effector CD8+ T cell generation (Curtsinger et al., 2003). IL-12 functions best when CD8+ T cells are exposed to peptide-MHC, signal 2, and IL-12 continuously from the onset of activation. However, IL-12 can be added as late as 18 hr in vitro and 24 hr in vivo after CD8+ T cell activation and still positively influence proliferation and survival of T cells. IL-12 may promote the survival of activated T cells by upregulation of the antiapoptotic protein Bcl-3 (Valenzuela et al., 2005) and/or by inhibiting activation of the proapoptotic enzyme caspase-3 (Palmer et al., 2001).

Accumulating evidence supports a direct role for IL-12 in facilitating optimal CD8+ T cell responses in vivo. For example, IL-12 functions as well as complete Freund's adjuvant (CFA) in promoting full activation of CD8+ T cells in an in vivo model of peptide immunization (Schmidt and Mescher, 1999). Interestingly, IL-12 promotes the peptide-stimulated response of wild-type CD8+ T cells that were placed in IL-12Rβ1-deficient mice (Schmidt and Mescher, 2002), suggesting a direct interaction of IL-12 with the responding T cells. Consistent with this idea, TCR-transgenic (TCR-tg) CD8+ T cells lacking both the IL-12 and IFN-αβ receptors undergo reduced in vivo expansion and differentiation compared to wild-type cells after *Listeria monocytogenes* infection (Z. Xiao and M. Meschser, personal communication). However, IL-12p35-deficient mice are capable of generating Ag-specific CD8+ T cell responses after lymphocytic choriomeningitis virus (LCMV) infection (Cousens et al., 1999). These data suggest that other cytokines may function as signal 3 for CD8+ T cell responses and that pathogen biology may dictate which cytokines are essential.

IFN-αβ as Signal 3

Marrack and colleagues identified IFN-αβ as capable of directly supporting the in vitro survival of in vivo-activated CD4+ and CD8+ T cells via a mechanism that does not involve upregulation of the antiapoptotic molecules Bcl-2 or Bcl-XL (Marrack et al., 1999). IFN-α is also capable of promoting optimal CD8+ T cell expansion and development of lytic capabilities after in vitro activation with peptide-MHC and B7.1, suggesting that IFN-αβ can function as signal 3 (Curtsinger et al., 2005). In contrast to these in vitro studies, the role of IFN-αβ in promoting the expansion of Ag-specific CD8+ T cells in vivo after infection is less clear. Nearly normal expansion of Ag-specific CD8+ T cells occurs following LCMV infection of IFN-αβR-deficient mice compared to wild-type mice (Cousens et al., 1999; Ou et al., 2001), although the proportions of Ag-specific CD8+ T cells generated to each epitope in the IFN-αβR-deficient mice are altered

(Cousens et al., 1999). Interestingly, and in contrast to these early results, IFN-$\alpha\beta$R-deficient P14 TCR-tg CD8$^+$ T cells adoptively transferred into wild-type hosts exhibit a severe defect in their ability to expand and generate memory populations after LCMV infection (Aichele et al., 2006; Kolumam et al., 2005). The small number of memory cells generated are maintained for at least 140 days after infection, indicating that the activity of IFN-$\alpha\beta$ is important for optimal T cell expansion, not in the maintenance of memory CD8$^+$ T cells (Kolumam et al., 2005). IFN-$\alpha\beta$R-deficient CD8$^+$ T cells demonstrate equal or greater rates of division compared to wild-type cells after LCMV infection; however, the IFN-$\alpha\beta$R-deficient CD8$^+$ T cells fail to accumulate, indicating a deficiency in survival of these cells in the absence of IFN-$\alpha\beta$ signaling (Aichele et al., 2006; Kolumam et al., 2005). In addition, recent studies show that IFN-$\alpha\beta$R-deficient TCR-tg CD4$^+$ T cells adoptively transferred into wild-type mice expand >99% less than wild-type tg T cells after LCMV infection (Havenar-Daughton et al., 2006). These data strongly argue that direct IFN-$\alpha\beta$R signals received by T cells serve as a critical signal 3 after LCMV infection. Although the discrepancy between the seemingly normal expansion after LCMV infection of IFN-$\alpha\beta$R-deficient mice and the reduced expansion of IFN-$\alpha\beta$R-deficient T cells has not been formally resolved, the high-level and protracted LCMV infection observed in the IFN-$\alpha\beta$R-deficient mice (Cousens et al., 1999) is a potentially important variable between the experimental systems.

However, there are a number of infectious model systems where IFN-$\alpha\beta$ signaling is dispensable for the expansion of Ag-specific T cells. IFN-$\alpha\beta$R-deficient P14 TCR-tg CD8$^+$ T cells exhibit only a modest defect in expansion and survival after infection with recombinant vaccinia virus expressing the LCMV glycoprotein (Aichele et al., 2006). Similarly, IFN-$\alpha\beta$ signaling is not required for the expansion of OT-II TCR-tg CD4$^+$ T cells (Havenar-Daughton et al., 2006), OT-1 TCR-tg CD8$^+$ T cells (Z. Xiao and M. Mescher, personal communication), or endogenous $OVA_{257-264}$-specific CD8$^+$ T cells (A. Jabbari, V.P.B., and J.T.H., unpublished data) after infection with *L. monocytogenes* expressing ovalbumin. To directly test the dependence on IFN-$\alpha\beta$ for signal 3 during different infections, adoptively transferred wild-type and IFN-$\alpha\beta$R-deficient OT-1 TCR-tg CD8$^+$ T cells were primed with OVA-coated DCs (controlled amount of Ag) at the same time that the recipient mice were infected with LCMV, vaccinia virus, vesicular stomatitis virus, or *L. monocytogenes* to induce inflammation. Wild-type cells expanded equally well after DC-peptide priming in the presence of all four types of infections, indicating that all of the infections tested generated one or more cytokines that could provide signal 3 to responding CD8$^+$ T cells. IFN-$\alpha\beta$R-deficient CD8$^+$ T cells exhibited the greatest defect in expansion when primed during LCMV infection, highlighting the dependence of CD8$^+$ T cells on IFN-$\alpha\beta$ for signal 3 during this infection (Thompson et al., 2006). These data further illustrate that pathogen biology is most likely critical in determining the specific requirement for signal 3 by Ag-specific T cells.

IFN-γ as Signal 3

IFN-γ is also able to promote optimal expansion of Ag-specific T cell populations after LCMV infection (Badovinac et al., 2000; Whitmire et al., 2005a, 2005b) or peptide immunization (Sercan et al., 2006). Reduced expansion of IFN-γR1-deficient cells compared to wild-type P14 TCR-tg CD8$^+$ T cells in the same host after LCMV infection indicates that IFN-γ acts directly on the responding CD8$^+$ T cells to promote their expansion (Whitmire et al., 2005b). Similarly, IFN-γR1-deficient CD4$^+$ TCR-tg cells fail to expand normally after LCMV infection (Whitmire et al., 2005a). In a separate study, wild-type CD8$^+$ T cells transferred into IFN-γ-deficient mice expanded 80% less than cells transferred into wild-type mice after immunization with peptide plus LPS (Sercan et al., 2006). However, similar to what has been observed with mice and T cells lacking IFN-$\alpha\beta$R, expansion of *L. monocytogenes*-specific CD4$^+$ and CD8$^+$ T cells in mice lacking IFN-γ or the IFN-γR1 is equal or greater than in wild-type mice, indicating that IFN-γ signaling in Ag-specific T cells is not required for expansion and that some other cytokine is capable of providing signal 3 to responding T cells after *L. monocytogenes* infection (Badovinac et al., 2000; J.S.H. and J.T.H., unpublished data).

Why Can So Many Inflammatory Cytokines Serve as Signal 3?

The collective theme is that a third signal delivered to Ag-specific T cells, in addition to peptide-MHC and signal 2, is required to promote optimal clonal expansion, perhaps by increasing survival of effector cells (Figure 2). This signal is not limited to one cytokine but can be delivered by at least three candidate cytokines in vitro and in vivo. The puzzle remaining is why optimal T cell expansion and survival is dependent on variable sources of signal 3 after different types of infection. Viruses, bacteria, and protozoan pathogens will likely activate a nonoverlapping spectrum of PRR, leading to unique patterns of inflammation. For instance, large amounts of IFN-$\alpha\beta$ are produced after LCMV infection, but virtually no IL-12 can be detected (Cousens et al., 1997; Orange and Biron, 1996; Welsh, 1978). In contrast, IFN-β mRNA is modestly upregulated (J.S.H. and J.T.H., unpublished data) but IL-12 protein (Auerbuch et al., 2004) and large amounts of IFN-γ mRNA and protein are produced after *L. monocytogenes* infection (Haring et al., 2005; Poston and Kurlander, 1991, 1992). In addition, the target cells and the outcome of infection (lytic for *L. monocytogenes* versus nonlytic for LCMV) may influence the nature of the inflammatory response. Thus, the combination of cellular responses to each pathogen most likely translates into a unique pattern of inflammation that dictates which cytokine provides the essential signal 3 required for optimal T cell responses.

The timing of cytokine signaling required for optimal expansion and survival is an additional area of interest. IL-12 functions best when T cells are continuously exposed to the cytokine in vitro but can be added to T cells at later times and still improve their ability to kill targets (Curtsinger et al., 2003). In mice or cells lacking IFN-$\alpha\beta$ or IFN-γ receptors, the T cells are without signals from these cytokines throughout the expansion phase. Because T cells lacking the IFN-$\alpha\beta$R divide but fail to survive (Kolumam et al., 2005), this suggests that IFN-$\alpha\beta$ may be required postpriming to stimulate survival of the proliferating T cells. In support of this idea, Ag-specific CD8$^+$ T cells moderately downregulate mRNA for

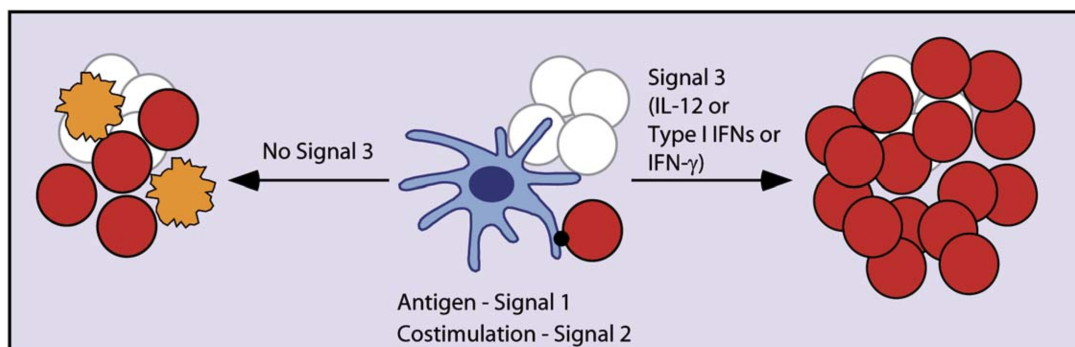

Figure 2. Signal 3 Is Required for Optimal CD8+ T Cell Clonal Expansion and Survival

Maximal expansion, function, and survival of effector CD8+ T cells depend on the action of cytokines that are shown to act as signal 3 during the early stages of the CD8+ T cell response. Although multiple cytokines (IL-12, type I IFNs, and IFN-γ) can serve as signal 3 for optimal CD8+ T cells in vitro, in vivo studies suggest that signal 3 after infection may be pathogen dependent.

both chains of the IFN-αβ receptor very early after infection with *L. monocytogenes*, but expression of both receptor chains is back to naive levels by 3 days after infection and remains stable throughout the expansion phase (J.S.H. and J.T.H., unpublished data).

In contrast, there is evidence that stimulatory IFN-γ signals may be required very early during the CD8+ T cell response. Ag-specific CD8+ T cells receive IFN-γ signals within 12 hr after infection with *L. monocytogenes* and then lose their responsiveness to IFN-γ via downregulation of IFN-γR2 until the end of their expansion phase (Haring et al., 2005). Thus, any positive effect that IFN-γ can have on the expansion of CD8+ T cells must be proximal to priming. Later during expansion, it may be important for T cells to evade signals delivered by IFN-γ in order to avoid the proapoptotic effects of this cytokine (Boehm et al., 1997; Refaeli et al., 2002).

In summary, although multiple cytokines can serve as signal 3 for optimal CD8+ T cell responses in vitro, the requirement for individual, pathogen-specific signal 3 is apparent after in vivo infection. Understanding the factors that determine which cytokines function as signal 3 during immune responses to infection will likely require a systems approach to analyze the complex interplay of inflammation and the responding CD8+ T cells after diverse infections. Furthermore, additional studies are necessary to fully determine the signal 3 requirements by responding CD4+ T cells that are necessary after different infections for optimal expansion and differentiation into memory CD4+ T cells. This information is likely to be critical in the choice of adjuvants to optimize both CD4+ and CD8+ T cell responses to vaccination.

Inflammatory Cytokines and T Cell Contraction

The expansion phase of Ag-specific CD8+ T cells is followed by an equally abrupt contraction phase, where 90%–95% of Ag-specific CD8+ T cells are eliminated. This substantial yet incomplete downsizing ensures that space and resources are available for CD8+ T cell responses against new pathogens while also maintaining a heightened ability to respond against previously encountered pathogens (memory). Although early models suggested that contraction is induced via activation-induced cell death (AICD), subsequent studies demonstrated normal contraction of Ag-specific CD8+ T cells lacking the death receptors (CD95 and TNFRI) implicated in classical AICD (Nguyen et al., 2000; Reich et al., 2000). More recent investigations have focused on regulation of pro- and antiapoptotic Bcl-2 family members, perhaps through modulation of common gamma-chain-utilizing cytokine receptors such as the IL-7R. For example, deletion of the BH3-only Bcl-2 family member Bim prevents the massive apoptosis of T cells observed after in vivo superantigen challenge (Hildeman et al., 2002) and also ameliorates contraction after HSV infection (Pellegrini et al., 2003). Although initial results revealed an excellent correlation between the fraction of IL-7R-expressing CD8+ T cells at the peak of expansion and the number of Ag-specific CD8+ T cells that survive contraction (Badovinac et al., 2004; Huster et al., 2004; Kaech et al., 2003), other studies demonstrate that expression of the IL-7R is not sufficient to prevent contraction under less inflammatory circumstances (Badovinac et al., 2005; Lacombe et al., 2005). Thus, the signals that regulate death of most, but not all, T cells present at the peak of the response and the precise death pathways involved remain to be elucidated.

After acute infection, the onset of the contraction phase generally starts as the pathogen is cleared. This correlation implies that the onset of CD8+ T cell contraction is regulated by somehow "sensing" the clearance of infection. In contrast to this notion, experiments over the last several years demonstrate that the onset and degree of contraction are independent of the magnitude of Ag-specific CD8+ T cell expansion, as well as the dose and duration of infection (Badovinac et al., 2002; Wherry et al., 2003a). These results suggest that early events after infection are also critical for "programming" CD8+ T cell contraction. Paradoxically, at least one of the inflammatory cytokines (i.e., IFN-γ) that regulate the expansion phase also influences the onset and degree of T cell contraction.

A substantial reduction in Ag-specific CD8+ T cell contraction occurs in mice lacking IFN-γ after infection with attenuated *L. monocytogenes* and LCMV (Badovinac et al., 2000). Ag-specific CD8+ T cell numbers remain elevated in IFN-γ-deficient mice for at least one year after *L. monocytogenes* or LCMV infection (Badovinac et al., 2000; unpublished data). Reduced contraction is still observed when IFN-γ-deficient mice are treated with

antibiotics to ensure complete clearance of attenuated *L. monocytogenes* infection (Badovinac et al., 2004), demonstrating that prolonged infection does not account for decreased CD8⁺ T cell contraction in the absence of IFN-γ. Therefore, IFN-γ influences the degree of contraction of CD8⁺ T cell responses after infection (Figure 3).

Expansion of Ag-specific CD8⁺ T cells in IFN-γ-deficient mice is normal or even enhanced depending on the epitope evaluated after attenuated *L. monocytogenes* infection but is reduced after LCMV infection (Badovinac et al., 2000). These experiments raise the interesting question of whether contraction is directly linked to the degree of expansion or is required for memory CD8⁺ T cell generation. Pretreatment of wild-type mice with antibiotics prior to attenuated *L. monocytogenes* infection allows the generation of fully functional CD8⁺ T cell memory in the absence of contraction (Badovinac et al., 2004). Inflammatory cytokines including IFN-γ are greatly reduced in the antibiotic-pretreated mice, and, consistent with a role for inflammation in regulating expansion, the magnitude of the CD8⁺ T cell response is also diminished (Figure 3). However, injection of CpG ODN to induce inflammation (including IFN-γ secretion) in the antibiotic-pretreated mice restores contraction without altering the magnitude of expansion. Together, these data suggest that inflammatory signals, rather than the magnitude of expansion, regulate CD8⁺ T cell contraction. In addition, these experiments demonstrate that contraction is not required for the generation of functional CD8⁺ T cell memory.

As discussed previously, IFN-γ mRNA and protein are markedly upregulated for 1–2 days after *L. monocytogenes* infection and then return to baseline. Consistent with this timing, Ag-specific CD8⁺ T cells receive IFN-γ signals within 12 hr after infection and then become refractory to further signaling by downregulating IFN-γR2 during the expansion phase (Haring et al., 2005). The fraction of Ag-specific CD8⁺ T cells that regain responsiveness to IFN-γ signals actually increases during contraction, an observation that is not consistent with the ability of this cytokine to directly kill effector cells. This hypothesis is further supported by the absence of IFN-γ mRNA in the spleen or IFN-γ protein in the serum during contraction (Haring et al., 2005). Although space limitations preclude a detailed discussion here, several studies suggest that IFN-γ also regulates contraction of Ag-specific CD4⁺ T cells (Chu et al., 2000; Dalton et al., 2000; J.S.H. and J.T.H., unpublished data). Together, these data suggest that IFN-γ signals delivered to responding T cells early after infection will influence the contraction program, as well as the expansion program, of CD8⁺ T cell responses. Thus, IFN-γ appears to play a pivotal role in CD8⁺ T cell homeostasis, with the capacity to enhance effector cell numbers and function but also to ensure appropriate contraction of these Ag-specific populations. The challenge is to identify the molecular pathways initiated by IFN-γ in T cells within hours after infection to regulate the contraction of Ag-specific T cells after many rounds of division.

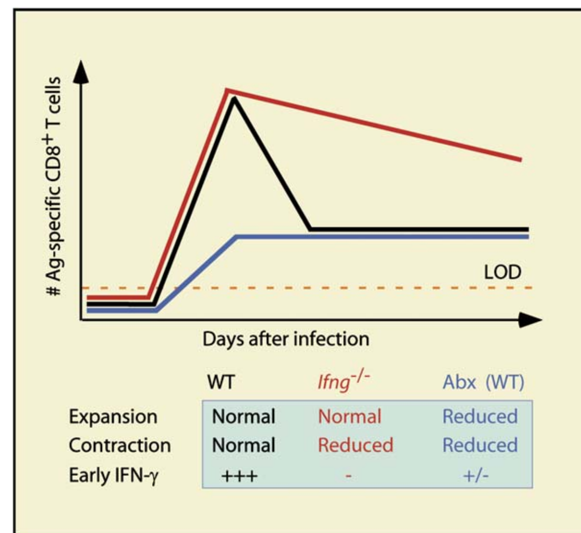

Figure 3. Modulation of CD8⁺ T Cell Expansion and Contraction Phases by IFN-γ

Normal expansion but reduced contraction of Ag-specific CD4⁺ and CD8⁺ T cells is observed after infection of IFN-γ-deficient mice with attenuated *L. monocytogenes* (IFN-γ-deficient; red line) compared to the severe contraction of CD8⁺ T cell responses in wild-type hosts (WT; black line). Antibiotic (Abx) pretreatment of IFN-γ-sufficient wild-type mice (Abx WT; blue line) before *L. monocytogenes* infection results in reduced expansion but no contraction of CD8⁺ T cells as well as substantially decreased levels of IFN-γ early after bacterial challenge.

CD8⁺ T Cell Memory

The 5%–10% of effector CD8⁺ T cells that survive the contraction phase initiate the memory pool, which can remain stable in number for life (Kaech et al., 2002b). However, memory CD8⁺ T cell maintenance is a dynamic process consisting of homeostatic proliferation that is balanced by death and involves changes in phenotype that may further shape the function, distribution, and per-cell protective capacity of memory CD8⁺ T cell populations (Masopust et al., 2001; Sallusto et al., 1999; Weninger et al., 2001). Due to their increased numbers, higher functional avidity, and lower signaling threshold required for reactivation, memory CD8⁺ T cells respond faster than their Ag-inexperienced naive counterparts and are able to provide heightened immunity after reinfection (Bachmann et al., 1999; Bertram et al., 2004; Kersh et al., 2003; Slifka and Whitton, 2001). In addition, memory CD8⁺ T cells are able to go through a second round of Ag-driven expansion that will result, depending on the dose of secondary challenge, in a further increase in memory CD8⁺ T cell numbers (Kaech et al., 2002b; Masopust and Ahmed, 2004). This latter property is critical for the success of prime-boost vaccination regimens (Woodland, 2004).

Even many weeks after initial stimulation, memory CD8⁺ T cells do not display a uniform phenotype, but they can be divided based on expression of surface markers into numerous subpopulations (for example, "effector memory" and "central memory"; Masopust et al., 2001; Sallusto et al., 1999) that differ in their anatomical distribution and ability to protect depending on the nature and route of infection (Roberts et al., 2005; Unsoeld et al., 2002; Wherry et al., 2003b). Some, but not all, markers that are used to distinguish Ag-experienced memory CD8⁺ T cells are also expressed on naive and/or effector CD8⁺ T cells. Furthermore, some of the key

markers (CD62L^{hi} and IL-2 production) used to distinguish primary "central memory" from effector and "effector memory" CD8^{+} T cells are downregulated on long-term secondary memory CD8^{+} T cells. However, we recently showed that, despite displaying a phenotype that is associated with impaired memory, secondary memory CD8^{+} T cells are able to provide better per-cell protective immunity and undergo expansion in numbers similar to primary memory cells after reinfection (Jabbari and Harty, 2006). Thus, identification of memory CD8^{+} T cells based on phenotype is problematic without precise knowledge of their Ag-exposure history. Although the heterogeneity in memory cell phenotype is daunting, additional studies to elicit the relationships between memory CD8^{+} T cell phenotype and function are warranted because this information may help tailor vaccine strategies to provide the most robust protection against specific pathogens. Finally, it should be remembered that the "noise" imprinted in the phenotype of memory CD8^{+} T cells likely represents an evolutionary strategy to maximize anatomical coverage and maintain readiness to defend against the widest range of pathogens.

Because the relationships between memory cell phenotype and function remain unclear, we prefer to define "good" memory CD8^{+} T cells based on their ability to (1) persist, (2) protect, (3) undergo vigorous expansion in response to pathogen rechallenge, and (4) generate higher numbers of memory cells after booster immunization. Next we will discuss recent evidence that inflammatory cytokines regulate the rate at which CD8^{+} T cells acquire memory characteristics and how vaccination can be manipulated to accelerate generation of Ag-specific CD8^{+} T cells that can respond to booster immunization within days of the initial stimulation.

Rate of Memory CD8^{+} T Cell Generation after Acute Infection

Over the last several years, it has become apparent that differentiation of naive to functional memory CD8^{+} T cells is the result of a program that is largely, although not exclusively, set in motion by early events after infection or vaccination (Badovinac et al., 2002; Kaech and Ahmed, 2001; Mercado et al., 2000; van Stipdonk et al., 2001; Wong and Pamer, 2001). This program is initiated by mature DCs that display pathogen-derived Ag and deliver costimulatory signals to T cells. Although not addressed in detail here, inflammation generated by infection clearly contributes to optimal maturation of DCs (Castellino and Germain, 2006; Heath and Carbone, 2001; Steinman, 2003). In addition, signals from other cell types such as CD4^{+} T cells may be essential to generate the most robust CD8^{+} T cell memory after vaccination and some infections (Bevan, 2004; Janssen et al., 2005; Shedlock and Shen, 2003; Sun and Bevan, 2003; Sun et al., 2004). Finally, the ability to express CD25, the high-affinity IL-2R, during CD8^{+} T cell priming is critical for secondary CD8^{+} T cell responses (Williams et al., 2006). Together, these data suggest that the program leading to CD8^{+} T cell memory is complex and dependent on the integration of multiple signals by the responding CD8^{+} T cells.

Ag-independent, functional CD8^{+} T cell memory populations are formed after acute infections. Successful clearance of infection and the resulting Ag-free environment may be required for development of fully functional memory CD8^{+} T cells (Wherry and Ahmed, 2004). However, the acquisition of memory CD8^{+} T cell characteristics, including the ability to vigorously respond to booster immunizations, by the populations of Ag-specific CD8^{+} T cells takes a month or more after clearance of acute infection (Kaech et al., 2002a) (Figure 4A). Although the cells that will survive contraction and seed the memory pool (memory precursors) may already be present at the peak of expansion, their ability to respond vigorously to booster immunization is likely masked by the numerically superior effector CD8^{+} T cell pool (Badovinac et al., 2005; Wong et al., 2004). However, even after elimination of the effector pool by contraction, early memory CD8^{+} T cells do not exhibit the same capacity to undergo secondary expansion as late memory populations (Kaech et al., 2003; V.P.B. and J.T.H., unpublished data).

Understanding why CD8^{+} T cells need time to develop "good" memory characteristics and whether this interval can be manipulated may be important in the design of optimal prime-boost vaccination protocols. Experiments from several systems suggest that the rate at which CD8^{+} T cells acquire memory characteristics may not be fixed. Reducing the stimulation of CD8^{+} T cells during infection with intracellular pathogens facilitates differentiation primarily into a CD62L^{hi} subset (van Faassen et al., 2005). Similarly, the conversion from CD62L^{lo} to CD62L^{hi} memory CD8^{+} T cells is faster after low-dose than high-dose *L. monocytogenes* infection (Wherry et al., 2003b). Decreasing the duration of *L. monocytogenes* infection by antibiotic treatment 24 to 48 hr after infection has minimal impact on expansion, contraction, and memory of Ag-specific CD8^{+} T cell numbers (Badovinac et al., 2002; Mercado et al., 2000). Interestingly, early termination of infection by antibiotic treatment results in a modest increase in the frequency of IL-2-producing memory CD8^{+} T cells (Badovinac et al., 2004; Williams et al., 2006). More recent studies show that progression to a CD8^{+} T cell memory phenotype (CD127^{hi}, CD43^{lo}, CD27^{hi}, IL-2 producing) is accelerated in antibiotic-treated compared to control-infected mice, as is the ability to proliferate in response to secondary *L. monocytogenes* booster immunization (V.P.B. and J.T.H., unpublished data). Therefore, manipulations to decrease the duration of infection in vivo may facilitate memory CD8^{+} T cell differentiation.

The time needed for CD8^{+} T cells to develop memory characteristics could be controlled by Ag display and/or inflammation, both of which are transient after acute infection (Figure 4A). Antibiotic treatment after *L. monocytogenes* infection diminishes both inflammatory cytokine (IFN-γ and TNF) production and the duration of Ag display (Badovinac et al., 2002; Mercado et al., 2000). Importantly, injection of CpG ODN to restore inflammation (Krieg, 2003; Takeda et al., 2003) in the antibiotic-treated mice prevents the rapid generation of memory phenotype CD8^{+} T cells and the ability to expand to secondary Ag challenge without changing the magnitude of expansion, contraction, or early memory cell numbers (V.P.B. and J.T.H., unpublished data). Furthermore, as discussed in the last section, pretreatment of mice with antibiotics followed by *L. monocytogenes* infection results in a 99.99% decrease in bacterial load at 24 hr after infection, diminishes IFN-γ production, and markedly impairs Ag-specific CD8^{+} T cell

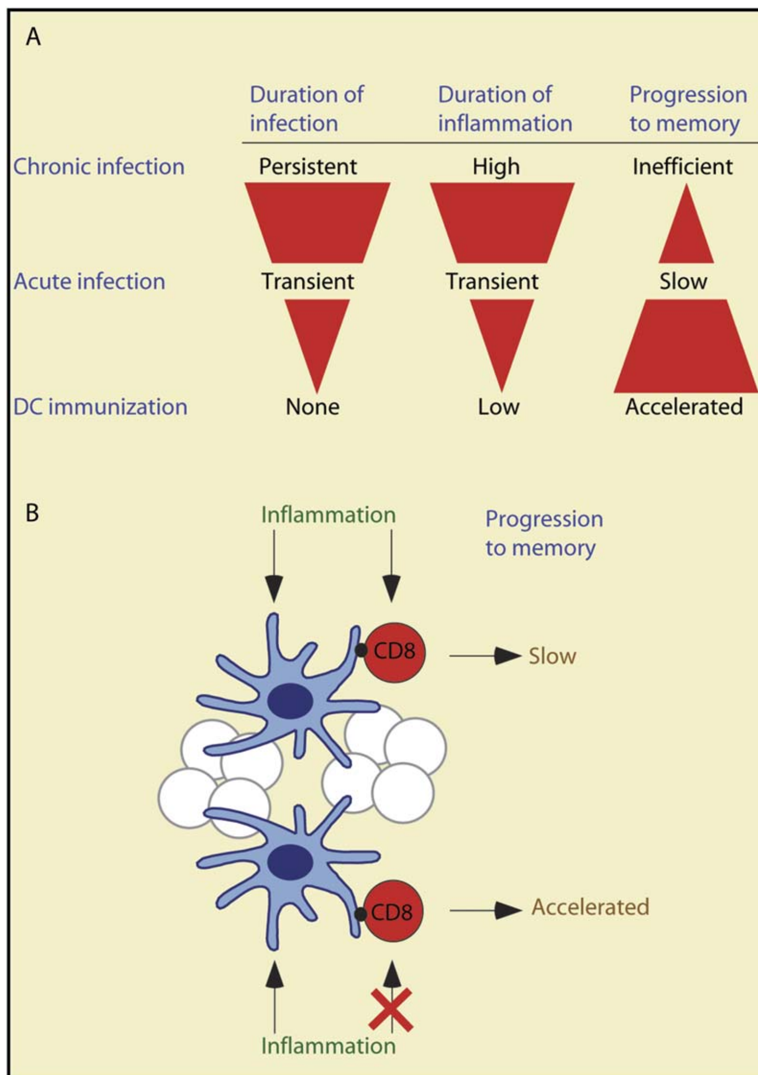

Figure 4. The Role of Inflammation in CD8+ T Cell Differentiation to Memory

(A) Depending on the nature of infection (chronic versus acute) or immunization (peptide-DC), CD8+ T cells exhibit differential progression to memory that depends on the degree and duration of infection and inflammation. During chronic infections, CD8+ T cells never attain all of the phenotypic and functional characteristics of long-term memory, while successful clearance of the pathogen after acute infections enables slow progression to fully functional CD8+ T cell memory. Naive CD8+ T cell activation after peptide-DC immunization occurs in the absence of infection or inflammation, and progression to CD8+ T cell memory is accelerated.

(B) Model: The rate of memory CD8+ T cell differentiation is influenced by the presence or absence of inflammatory stimuli received by responding CD8+ T cells.

expansion. Follow-up studies reveal that CD8+ T cells primed in antibiotic-pretreated mice develop phenotypic (CD127^{hi}, CD27^{hi}, CD43^{lo}) and functional (IL-2 production and vigorous proliferation after secondary challenge) characteristics of long-term memory CD8+ T cells within one week after immunization (V.P.B. and J.T.H., unpublished data). This accelerated generation of CD8+ T cells with memory characteristics correlates with substantially decreased amounts of proinflammatory cytokines and chemokines (IFN-γ, TNF, IL-6, IL-12, IL-10, RANTES, MIP-1α, G-CSF) during the first 24 hr after infection compared to control-infected mice (Badovinac et al., 2004; V.P.B. and J.T.H., unpublished data). Although it remains unknown how DCs receive maturation signals in the absence of inflammatory cytokines, these data suggest that the extent and duration of infection-induced inflammation influence the rate of memory CD8+ T cell generation (Ahmed and Gray, 1996; Masopust et al., 2004).

CD8+ T Cell Memory after DC Immunization

Immunization with in vitro-matured peptide-loaded DCs provides a setting where CD8+ T cells receive the full complement of mature DC-mediated signals in the absence of infection- or adjuvant-induced inflammation (Hamilton and Harty, 2002). Consistent with the idea that inflammation influences the rate of memory CD8+ T cell generation, we observe rapid differentiation of CD8+ T cells from naive to effector to memory after immunization of mice with in vitro-matured peptide-loaded DCs (Badovinac et al., 2005). As early as 4 to 6 days after peptide-DC immunization, the majority of Ag-specific CD8+ T cells express markers (CD127^{hi}, CD27^{hi}, CD43^{lo}, IL-2 production) of long-term memory. These early memory cells are able to vigorously proliferate in response to a variety of booster regimens, leading to a rapid elevation in the numbers of secondary effector CD8+ T cells, substantially elevated secondary memory cell numbers, and enhanced protective immunity. DC immunization does not evoke systemic production of inflammatory cytokines and also results in a relatively short window of "functional" Ag presentation as detected by proliferation of injected "sensor" naive TCR-tg T cells. Coinfection of mice with *L. monocytogenes* or CpG ODN treatment to induce inflammation prevents the accelerated naive-to-memory CD8+ T cell transition

after peptide-DC immunization without changing the magnitude of Ag-specific CD8$^+$ T cell expansion. These data strongly argue that it is inflammation and not the duration of Ag display that regulates the rate at which CD8$^+$ T cells acquire memory characteristics (Figure 4A).

Understanding how inflammation controls the rate of memory CD8$^+$ T cell generation will require knowledge of the specific cytokines involved and whether these cytokines are (1) acting on DCs to control programming signals delivered to the CD8$^+$ T cells, (2) acting directly on the responding CD8$^+$ T cells, or (3) acting indirectly via other cell types. Several experiments suggest that inflammation might act on the responding CD8$^+$ T cell populations (Badovinac et al., 2005) (Figure 4B). Accelerated memory CD8$^+$ T cell differentiation occurs when CpG ODN is used for in vitro maturation of DCs. In contrast, CpG ODN treatment in vivo prevents the generation of early memory CD8$^+$ T cells without altering the lifespan of injected DCs, the duration of functional Ag presentation, or the magnitude of CD8$^+$ T cell expansion in vivo. Treatment with CpG ODN as late as three days after DC immunization, a time when DC-mediated Ag presentation is undetectable, still prevents early acquisition of memory characteristics by responding CD8$^+$ T cells, suggesting that CpG ODN (or CpG-induced inflammation) is not acting through the injected DCs. Importantly, CpG ODN treatment at the time of DC immunization does not prevent rapid memory CD8$^+$ T cell generation in IFN-γR2-deficient mice. These data suggest that IFN-γ-mediated signals received by responding CD8$^+$ T cells shortly after immunization play an important role in controlling the rate of memory CD8$^+$ T cell differentiation. Together, these studies suggest that vaccination strategies that allow maturation of DCs but limit inflammatory signals such as IFN-γ to the responding T cells may facilitate progression to "good" memory and decrease the interval required for increasing Ag-specific CD8$^+$ T cell effector and memory numbers by booster immunization.

Inefficient Memory CD8$^+$ T Cell Generation after Chronic Infections

Acute infections and DC immunization both generate "good" memory CD8$^+$ T cell populations, although at different rates. In contrast, multiple studies show that chronic infections lead to inefficient or poor-quality CD8$^+$ T cell memory (Klenerman and Hill, 2005; Wherry et al., 2004) (Figure 4A). CD8$^+$ T cells at late time points in chronically infected mice are "exhausted" (deleted or functionally impaired) and are unable to provide sterilizing immunity (Moskophidis et al., 1993; Wherry et al., 2003a; Zajac et al., 1998). In addition, memory CD8$^+$ T cells from chronically infected mice cannot persist after transfer to an Ag-free environment, partly due to their inability to undergo IL-7- and IL-15-driven homeostatic proliferation (Klenerman and Hill, 2005; Wherry et al., 2004). Memory CD8$^+$ T cells from chronically infected mice exhibit increased expression of programmed death 1 (PD-1) compared to memory CD8$^+$ T cells after acute LCMV infection. Interestingly, PD-1 is relevant for maintaining the dysfunction of memory cells in chronically infected mice because blocking the interaction with its ligand, PD-1L, enhances viral control, increases CD8$^+$ T cell function (cytokine production and cytotoxicity), and restores proliferation in response to viral Ag (Barber et al., 2006). Although this study (Barber et al., 2006) suggests possible strategies to facilitate viral control during chronic infections, it is not well understood why CD8$^+$ T cells that develop during prolonged exposure to Ag (pathogen) never attain all of the phenotypic and functional hallmarks of "good" memory cells. Given the evidence that inflammation regulates the rate at which CD8$^+$ T cells acquire memory characteristics, it is perhaps not surprising that dysfunction of CD8$^+$ T cells in at least some mouse and human chronic infections (e.g., HIV, HBV, and *Trypanosoma cruzi*) also correlates with persistent inflammation (Decrion et al., 2005; Leavey and Tarleton, 2003; Maini et al., 2000).

Summary and Future Directions

In addition to their role in stimulating antimicrobial defenses and the Ag-presenting capacity of host APCs, inflammatory cytokines act directly on responding T cells to affect all phases of the T cell response to infection. Depending on the pathogen, and likely on the adjuvant chosen for vaccination, signals from one or more inflammatory cytokines are required for proliferation and survival of the responding T cells, so that optimal expansion in effector T cell numbers can occur to defend against infection. Somewhat paradoxically, at least one of the inflammatory cytokines (IFN-γ) required for optimal T cell expansion also ensures the appropriate contraction in numbers of Ag-specific T cells. Finally, inflammation regulates (delays) the rate at which Ag-specific CD8$^+$ T cells acquire important memory characteristics after infection and vaccination. Together, these data suggest that the type, magnitude, and duration of inflammation allow the responding T cells to sense the degree of "danger" associated with a specific Ag encounter and, in response, to potentially modify their expansion and contraction and maintain heightened immunity by delaying their transition from an effector population.

Clearly, strong adjuvants that elicit inflammation and provide signals to mature DCs improve the magnitude of the T cell response against nonreplicating vaccine agents. However, by mimicking infection, adjuvant-induced inflammation also causes substantial contraction and delays the acquisition of memory characteristics, including the ability to respond to booster immunization, by the responding T cell populations. An ideal vaccine may in fact evoke modest expansion in Ag-specific CD8$^+$ T cell numbers but rapid progression to memory with limited contraction, thus reducing the interval between initial priming and booster immunizations required for optimally protective secondary memory responses. The challenge will be devising vaccination regimens that promote DC maturation while limiting exposure of the responding T cells to inflammatory cytokines. Existing and emerging mouse models of infection, in combination with the ability to correlate phenotype with function of Ag-specific T cells, provide high-resolution systems to test these ideas in the context of vaccination. In addition, the most interesting future studies will address the mechanisms by which inflammatory cytokine signals delivered to Ag-specific CD8$^+$ T cells within hours of their initial stimulation can control the fate of these cells after many subsequent cell divisions.

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