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Once a killer, always a killer: from cytotoxic T cell to memory cell

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Summary

The control of the differentiation pathways followed by responding CD8⁺ T cells to produce protective memory cells has been intensely studied. Recent developments have identified heterogeneity at the effector cytotoxic T-lymphocyte level within which a *bona fide* memory cell precursor has emerged. The challenge now is to identify the cellular and molecular factors that control this developmental pathway. This review considers aspects of the regulation of the induction of effectors, the transition of effectors to memory cells, and the dynamics of the memory population.

Keywords

T cells; infectious diseases; cytokines; cytokine receptors; cell differentiation

Introduction

Induction of cytotoxic activity by CD8⁺ T cells has long been a topic of immunologic interest. Ever since cytotoxic activity was initially identified using alloantigen-activated thymus-derived cells (1,2), the process of cell-mediated cytotoxicity has been studied in much detail. Many of the earliest studies examined cytotoxic activity from the point of view of transplantation, because cytotoxic activity poses a significant obstacle to successful transplantation. Of course, the potential for cytotoxic activity to mediate tumor rejection is a major focus of cytotoxic T-lymphocyte (CTL) research. Once the exquisite specificity of CTL was clarified (3,4), the possibility of CTL induction by vaccination to prevent infectious disease and limit tumor progression has been further explored. Furthermore, the description of cytotoxic granule exocytosis and identification of cytotoxic effector molecules, such as perforin and granzymes (5), began the definition of the molecular basis of CTL and natural killer cell-mediated cytotoxicity. Nonetheless, much still remains to be learned regarding the cytotoxic process. This includes transcriptional, translational, and post-translational control of components of the cytotoxic machinery. In addition, the cellular mechanisms and even the precise array of proteins utilized by CTLs to kill antigen-bearing target cells are far from completely defined. More recently, additional effector molecules have been identified. These include granulysin, which exhibits lytic activity toward tumors and bacteria (6). Thus, the armamentarium that CTLs bring to bear on their opponents is complex, and the governance of the components of the system continues to be analyzed.

The linkage between CD8⁺ memory T cells and the induction of cytotoxic activity has been known for many years. In the early days of CTL research, ‘memory’ CD8⁺ T cells were

generated *in vitro* by resting allogeneic mixed lymphocyte reaction (MLR) cultures (7–10). In fact, these cultures mimicked to a reasonable extent what we now know from more sophisticated approaches. Responding MLR-induced T cells underwent initial blastogenesis followed by a contraction/death phase and a return to what appeared to be small, resting lymphocytes with low cytotoxic activity. Upon restimulation with the correct major histocompatibility complex (MHC), rapid reactivation of the MLR-induced ‘memory’ cells occurred with the induction of cytotoxic activity. Thus, the concept of ‘resting’ memory CTLs emerged. Since that time, our ability to detect and analyze antigen-specific effector and memory T cells at the cellular and molecular levels has improved by leaps and bounds because of many technological innovations including multicolor flow cytometric analysis and cell sorting, T-cell receptor (TCR) transgenic mice (11,12), adoptive transfer of TCR transgenic cells (13), gene-targeted knockout mice (14–18), conditional knockout mice using cre-recombinase (19), and MHC class I and class II tetramer technology (20,21). These technological advances have enabled us to garner a much more in depth knowledge of CD8⁺ T-cell responses and the relationship of naive, effector, and memory CD8⁺ T cells. Therefore, this review focuses on the developmental linkage between the induction of effector function and the generation of memory CD8⁺ T cells.

Induction of effector function is rapid and linked to memory T-cell development

Whether a naive CD8⁺ T cell transits through a requisite effector CTL stage prior to developing into a memory cell has been a long-standing question (22). The importance of the question is certainly germane to developmental immunology, but knowing the pathway of memory T-cell development could also allow clinically relevant manipulation of the system. An early study using TCR transgenic cells in an adoptive transfer system concludes that memory CD8⁺ T cells could be derived from effector cells (23). Subsequently, results from *in vitro* studies show that limited cell division is linked to the induction of CTL activity and formation of memory CD8⁺ T cells (24). Our work and that of others has also shown a tight linkage between induction of effector function and the generation of memory in both CD4⁺ and CD8⁺ T cells responding to infection. Limiting the level of inflammation or the strength of TCR signaling during the initiation of the T-cell response still allows effector induction and the magnitude of the effector response correlates with the level of memory development (25–27). The underlying problem with these studies is that they did not make definitive lineage designations and therefore were unable to accurately determine the fate of a given single cell. Thus, the bulk of the CTL population may exhibit cytotoxic abilities, but a small subset of cells lacking effector function may be obscured by the larger CTL population, and this small population of cells lacking cytotoxic abilities may preferentially survive to become memory cells. In addition, if sufficient ‘signal strength’ is related to both efficient effector induction and memory development, then again, the evidence remains only correlative.

Defining the naive to effector to memory transition

Attempts at addressing the issue of the precursor–product relationship between effector and memory CD8⁺ T cells have been made in a variety of ways over the past several years. For example, a recent report suggests that asymmetric division very early during the response results in development of separate effector and memory lineages (28). Thus, following initial activation, the first cell division produces one daughter cell with long-lived memory characteristics and another with short-lived effector abilities. The data definitively show that asymmetric division occurs as a result of engagement of the CD8⁺ T cell with an antigen-bearing antigen-presenting cell (APC). Less obvious is how the lineage relationships delineated after the initial divisions would be maintained throughout the remainder of the primary response leading into early memory development. It is not clear how downstream or serial

interactions of responding CD8⁺ T cells with antigen-bearing APC, which most certainly occur (26,29–31), would affect such a developmental scheme. This system also employed adoptive transfer of large numbers of clonal, high avidity CD8⁺ T cells, a necessary evil for this type of study but one that is known to affect response outcomes and memory development (32,33). This scenario of effector/memory dichotomy at the first cell division is also hard to reconcile with what is now known about effector subset heterogeneity (34), but nonetheless such a process cannot be categorically ruled out for a subset of responders. The fact that a subset of human and mouse memory CD8⁺ T cells, termed effector memory cells (T_{EM}) exhibit direct *ex vivo* lytic activity and constitutively express granzymes and perforin (35–37), can be used to argue that such cells are likely derived from effector cells. But what about the remainder of the memory population, the ‘central’ memory subset (T_{CM}) that does not exert constitutive effector function? Did these cells also transit through an effector phase? Several lines of evidence suggest that the answer is yes.

More recent support for a CTL memory differentiation model whereby a naive cell must pass through an obligatory cytotoxic effector stage before becoming a memory cell comes from a recent study using a genetic tagging scheme to study the effector cell to memory cell pathway (38). In this system, effector cells are indelibly marked by virtue of cre-recombinase-mediated activity controlled by the granzyme B promoter. Thus, when CD8⁺ T cells are activated in response to infection with influenza virus, cre-recombinase is expressed which then cleaves a sequence encoding a stop codon present in the targeted and ubiquitously expressed Rosa locus thereby allowing expression of yellow fluorescent protein (YFP). These events result in the long-term expression of YFP in any cell that had previously expressed granzyme B and therefore cre-recombinase. The results indicate that memory CD8⁺ T cells are derived from cells that had been transcriptionally active at the granzyme B locus. Thus, upon challenge a proportion of the secondary responders express YFP, identifying them as memory T cells derived from the primary response that had transited through an effector phase. However, this study does not prove that all memory cells are derived from effector cells as the system is inefficient and not all granzyme expressing primary effector cells express cre-recombinase. Yet, the findings delineate the stochastic nature of the CD8⁺ T-cell response with regard to initial events specifying downstream consequences.

It may also be worthwhile to consider what defines an effector CD8⁺ T cell. Historically, mediating lytic activity identified a CD8⁺ T cell as an effector. Later, the ability to produce cytokines became an additional hallmark of effector function. For the most part, lytic activity and cytokine production are coincident, although there are exceptions (39,40). Staining for intracellular proteins such as perforin or granzyme B that are known to be involved in lytic activity is oftentimes used as a surrogate marker for effector function. In addition, assays measuring degranulation are also employed to identify putative effectors (41). However, granzyme B, degranulation, or other surrogates may not always faithfully correlate with effector function. For example, granzyme A and B may not always participate in lytic activity (42). Similarly, while effector and memory cells rapidly degranulate when TCR is triggered, the contents of the cytotoxic granules are deterministic of the level of cytolytic function (43). Cytotoxic granule contents can also mediate protection against infection in unconventional ways such as the recently described action of granzyme B on inactivation of a herpes virus protein that is essential to pathogenesis (44). Granulysin, which has direct lytic activity on microbes and cells, is another granule component and is made by human, but not mouse CD8⁺ T cells and NK cells (6,45). Therefore, whenever possible, direct measurement of cytolytic activity is preferable to the use of detection of surrogate molecules or processes.

Arguments have also been made with respect to the relationship between effector CD4⁺ T cells and memory development (46). Although this issue will not be addressed in detail in this review, the authors of a recent study that employed a clever genetic approach also concluded

that memory CD4⁺ T cells can be derived from interferon- γ -producing primary effectors (47), but whether this is also true for T-helper 2 (Th2), Th17, and T-follicular helper cells remains unresolved. Thus, both effector CD4⁺ and CD8⁺ T cells retain the ability to differentiate into memory cells.

Early events during effector priming initiate memory development

The quest for the identity of a true memory precursor continues although significant progress in the characterization of early events specifying memory development has been made in recent years. The possibility that a subset of naive CD8⁺ T cells are predestined to become memory cells or that heterogeneity at the naive cell level might affect effector versus memory cell development has been discussed over the years. However, a recent study shows that predestiny or heterogeneity at the naive CD8⁺ T-cell level is not deterministic, because a single naive CD8⁺ T cell upon challenge with *Listeria monocytogenes* (LM) gives rise to heterogeneous effector and memory populations (48). Thus, stochastic events downstream of the initial activation of a single naive CD8⁺ T cell must determine which pathway a particular daughter cell chooses to follow into effector and memory subset development. It should be noted that the transferred naive CD8⁺ T cell is of course of a single avidity and a normal population of naive responders would be heterogeneous at least in terms of avidity. Additionally, the time at which a naive T cell enters the response may also affect the differentiation characteristics of that cell (49,50). Moreover, under normal circumstances, the precursor frequency of naive CD8⁺ T cells controls competition between clones and affects response outcomes (51). Thus, multiple factors at the cellular level are important for controlling the complex orchestration of the naive to effector cell transition.

Identification of memory CD8⁺ T-cell precursors has been a longstanding goal in the field. Our earlier work shows that expression of IL-7R α by CD8⁺ T cells responding to virus infection is critical for memory development (52). IL-7R α expression correlates with enhanced or sustained expression of intracellular anti-apoptotic survival molecules including bcl-2 (52, 53). However, a recent report showed that IL-7R α expression alone is not deterministic of memory development, because overexpressing IL-7R α in responding CD8⁺ T cells did not lead to enhanced memory generation (54). IL-7 may also not be essential for memory CD8⁺ T-cell development (55), suggesting that thymic stromal lymphopoietin, whose receptor consists of IL-7R α and a type-specific β -chain (56), may play a role in memory development. More recently, the combination of IL-7R α expression along with the expression of killer cell lectin-like receptor G1 (KLRG1) has been used in seminal studies defining heterogeneity at the level of effector CD8⁺ T cells (57,58). IL-7R α ^{high} KLRG1^{low} cells represent memory precursor effector cells (MPECs), while cells lacking IL-7R α and expressing KLRG1 represent short-lived effector cells (SLECs). Our work (J.J. Obar and L. Lefrançois, unpublished data) and that of others (34,59) identified an additional subset, termed early effector cells (EECs) that lack both KLRG1 and IL7R α expression. Transfer of purified EECs results in the development of MPEC and SLEC, identifying EECs as the earliest identifiable memory and effector precursors. Furthermore, we hypothesize that the EEC population likely comprises a heterogeneous population, possibly at the genetic level (i.e., epigenetic modifications), that perhaps contains cells already committed to the MPEC and SLEC lineages. Additional support for the linear differentiation model comes from the demonstration that nearly all cells of the EEC subset, as well as both the SLEC and MPEC subsets derived from the EEC population, express granzyme B (Fig. 1). Furthermore, SLECs and MPECs are also able to produce cytokines and mediate cytotoxic activity (57,58).

While KLRG1 serves as a useful marker to identify effector subsets, the functional relevance of KLRG1 expression *in vivo* is yet to be determined. KLRG1 was originally identified as a molecule expressed by rat mast cells and was termed mast cell function-associated antigen

(MAFA) (60,61). In these studies, KLRG1 was shown to inhibit immunoglobulin E (IgE)-mediated mast cell activation (61,62). Subsequently, the gene encoding the human ortholog of MAFA was identified in the NK cell gene complex and was shown to be expressed in human basophils, mast cells, and NK cells (63). KLRG1 is a C-type lectin and a type-II transmembrane protein with an immunoreceptor tyrosine-based inhibitory motif (ITIM) present in the cytoplasmic tail (63). Further work demonstrates that KLRG1 is expressed by replicatively senescent mouse and human effector/memory CD8⁺ T cells (64,65). As the SLEC population responds poorly to secondary infections (57,58), does KLRG1-E-cadherin interaction limit the proliferative capacity of cells expressing this molecule? Or does KLRG1 expression participate in the process of the overall genetic programming of SLEC that serves to limit their replicative abilities? Thus far, a KLRG1-deficient mouse has not been described. However, a recent analysis of human CTLs shows that KLRG1-mediated events maintain active negative signaling through interaction with E-cadherin (66). Blocking the binding of KLRG1 to E-cadherin resulted in enhanced Akt phosphorylation, proliferation, and induction of cyclins (66). Furthermore, KLRG1 has been shown to inhibit IL-2 production from T-cell hybridomas when engaged by E-cadherin (67). These experiments were performed with highly differentiated CD28⁺CD27⁺ CD8⁺ T cells (66) or T-cell hybridomas (67), thus whether KLRG1 plays a similar negative regulatory role in primary effector CD8⁺ T cells *in vivo* remains to be tested. Additionally, KLRG1 may mediate 'reverse' signaling through E-cadherin in APCs, which results in limited production of inflammatory cytokines (IL-6 and tumor necrosis factor- α) and enhanced IL-10 production (67). Nonetheless, the available results imply that negative regulation via KLRG1 and E-cadherin is an ongoing event that serves to control the magnitude and/or the longevity of the effector phase of the CD8⁺ T-cell immune response, particularly under inflammatory conditions.

Regulation of the dynamics of effector subset composition

Multiple signals are required to mount a productive CD8⁺ T-cell response to infection or vaccination. The 'three-signal' model accounts for signaling via (i) the TCR; (ii) CD28 or perhaps other costimulatory molecules; and (iii) receptors for inflammatory cytokines, particularly IL-12 and type I IFN α (68). It is now clear that IL-12 and IFN α are potent growth factors for effector CD8⁺ T cells (69–72). Moreover, the importance of IFN α versus IL-12 in a given response is dictated by the pathogen. Thus, the CD8⁺ T-cell response to lymphocytic choriomeningitis virus (LCMV) infection is highly IFN α dependent, and this infection induces large amounts of IFN α . Whereas CD8⁺ T-cell responses to infection with vesicular stomatitis virus (VSV), LM, or vaccinia virus (VV) are less IFN α dependent, and these infections induce lower levels of IFN α . Nevertheless, IFN α still provides a growth advantage to CD8⁺ T cells responding to these pathogens (70). Furthermore, both IFN α and IL-12 are required for optimal expansion of CD8⁺ T cells responding to LM or VSV infection (71,73, J.J. Obar and L. Lefrançois, unpublished results).

How both IFN α and IL-12 control CD8⁺ T-cell effector subset differentiation has been recently examined. While the overall expansion of CD8⁺ T cells is decreased when the CD8⁺ T cells lack IFN α or IL-12 receptors (71), both cytokines appear to preferentially enhance SLEC development (57,74; J.J. Obar and L. Lefrançois, unpublished results) (Table 1). The loss of SLEC results in concomitant increases in MPEC, but interestingly an increase in EEC also occurs. The latter finding may indicate that IL-12 and IFN α could play a role in differentiation of EEC toward the SLEC pathway (Fig. 2). The fact that IL-12 action is needed very early in the response (74) supports this possibility. Both IL-12 and IFN α likely also promote survival, as well as expansion of the effector CD8⁺ T cells (69–73,75,76). Additionally, IFN γ produced during infection regulates the expansion, survival, and differentiation of CD8⁺ T cells. IFN γ can provide signals directly to the CD8⁺ T cell enhancing their proliferation and survival (77). Furthermore, IFN γ production appears to promote IL-12 production from APCs, which

thereby enhances the CD8⁺ T-cell response and subsequent differentiation into SLECs (74). Thus, inflammatory cytokines have a dramatic impact on the overall expansion, survival, and differentiation of CD8⁺ T cells.

One potential means of enhancing the efficacy of vaccines is the use of an adjuvant, such as Toll-like receptor (TLR) ligands (78). TLR ligands are known to enhance immune responses by enhancing APC maturation enabling better antigen presentation and the secretion of numerous inflammatory signals (79). As such, the inflammatory signals resulting from TLR ligand administration can modulate the EEC/MPEC/SLEC ratios (57,74,80). For example, TLR9 ligation induces the production of IL-12 and/or IFN α , thereby enhancing SLEC development (57,74,80), but whether this holds true for other TLR ligands remains unknown. Thus, depending on the scenario, the use of adjuvants to enhance vaccination could be counterproductive. While the primary response induced by an adjuvant may be robust, the preferential generation of SLECs could limit protective memory CD8⁺ T-cell development. Therefore, a balance must be reached such that an appropriate level of inflammation is induced to drive a sufficiently robust productive response, but also such that the inflammation is limited to the extent that memory development is not impaired. This principle is demonstrated by vaccination with peptide coated activated dendritic cells (DCs), which results in the preferential and rapid development of IL-7R α^{high} memory-like cells. The resulting cells are able to quickly respond to challenge, showing that they functionally resemble *bona fide* memory cells (81). A similar process occurs when LM infection is limited by antibiotic treatment. That is, although primary CD8⁺ T-cell expansion is blunted in this situation, IL-7R α^{high} memory-phenotype cells rapidly appear, and these cells are able to respond to recall antigen (27). Limiting LM infection also limits SLEC development (57). More recently, a low dose of rapamycin, which is typically used as an immunosuppressive drug, was shown to enhance MPEC formation through limiting inflammatory signals received by the CTL (82). Furthermore, regulation of AMP-activated kinase (AMPK) signaling by administration of metaformin demonstrates a similar increase in memory formation (83). Both of these novel 'adjuvants' (rapamycin and metaformin) appear to work by limiting the ability of the responding CD8⁺ T cells to sense the inflammatory milieu, supporting the notion that a highly inflammatory environment supports SLEC development, while low inflammatory environments promote MPEC formation. Importantly, the use of rapamycin and metaformin did not impact the overall expansion of CD8⁺ T cells, making them extremely attractive as adjuvants.

In addition to IL-12, IFN α/β , and IFN γ , multiple other factors control effector subset development (Table 1). For the most part the factors that have been defined appear to be those that control responses to inflammation and therefore effect SLEC development or survival. Furthermore, the transcription factors through which inflammatory cytokines may be operating have also begun to be identified. For example, the expression level of the transcription factor T-bet helps determine the SLEC/MPEC ratio (57). High levels of IL-12 drive heightened T-bet expression resulting in enhanced SLEC development. Conversely, the reduced T-bet levels present when inflammation is limited favors MPEC generation (57). Additionally, Blimp1 has recently been identified as another transcription factor that promotes SLEC differentiation (84,85). We have recently demonstrated that IL-2 enhances SLEC differentiation (86), which could be mediated via upregulation of Blimp1 (87). Thus, we are beginning to garner a more complete understanding of how inflammatory mediators work to influence the development of an immunologic response.

IL-7 and IL-15 γ_c cytokine involvement in effector and memory CD8⁺ T-cell development and survival

Cytokines that signal via receptors containing the common γ chain (γ_c) are critically important for induction and maintenance of effector and memory CD8⁺ T cells. In particular, the role of

the cytokines IL-2, IL-7, and IL-15 in CD8⁺ T-cell biology has been studied in substantial detail (88). As discussed above, the expression of IL-7R α , though not necessarily IL-7, is important primarily after initial CD8⁺ T-cell expansion and is essential for memory cell development and survival. Nevertheless, although the overall magnitude of the response is normal in the absence of IL-7, the response of adoptively transferred OT-I cells responding to LM-ova infection is heavily skewed toward SLEC and away from MPEC generation/survival (89). Recent reports have demonstrated that γ_c cytokine activity can be enhanced by precomplexing the cytokine with cytokine-specific antibodies (IL-2 and IL-7) or soluble high-affinity receptor (IL-15) (90–92). Treatment of mice with IL-7/ α IL-7 complexes during the contraction phase of a CD8⁺ T-cell response results in preferential survival of MPEC (89). This result indicates that antibody-complexed IL-7 selectively acts on those cells expressing IL-7R α . In case of IL-15, the action of the cytokine *in vivo* is mediated via the unusual process of transpresentation (93). In the process of transpresentation, CD8⁺ T cells responding to IL-15 need not express the high-affinity IL-15R α , but must express the IL-2/15R β and γ_c (94–97). Cells producing IL-15 and concomitantly expressing IL-15R α , such as DCs, transpresent IL-15 to apposing cells (95,96,98–101). Precisely how IL-15 and IL-15R α are controlled during a dynamic infection-driven CD8⁺ T-cell response is not entirely clear. In any case, the contraction phase of the response following infection of IL-15^{-/-} mice with either virus or bacteria is dramatically enhanced, beginning earlier and resulting with only a small number of cells surviving the crash (89,102). Interestingly, despite the requirement for IL-15 in the proliferative maintenance of CD8⁺ memory T cells, SLECs appear to be especially sensitive to the action of IL-15 and are more rapidly lost than MPECs in the absence of IL-15 (57,89). Treatment with superagonistic IL-15/IL-15R α or IL-2/ α IL-2 complexes also enhances SLEC formation (89). The dichotomy in IL-15 action on SLECs versus MPECs may be related to the effect of the cytokine on survival versus proliferation of one subset versus another. Additionally, this dichotomy could be a result of the sensitivity of SLECs to transforming growth factor (TGF)- β -induced apoptosis, which can be rescued by IL-15 (59). IL-15 is responsible for homeostatic proliferation of memory CD8⁺ T cells, while IL-15 during the primary response may be more involved in survival of SLECs and opposing the actions of TGF β (Fig. 2). Indeed, SLECs proliferate poorly in response to IL-2/ α IL-2 or IL-15/IL-15R α complexes, suggesting that these agents are more important for assisting in survival of this subset (89).

The link between CD4⁺ T-cell help, costimulation, and IL-2 activity in regulating CD8⁺ T-cell responses

Priming of CD8⁺ T cells can occur in multiple ways depending on the infection or vaccination scheme. In some cases, the requirement for particular cytokines or costimulators may be bypassed via activation and involvement of alternate pathways (103–105). While some CD8⁺ T-cell responses require CD4⁺ T-cell help, others apparently do not. This is also true for certain costimulators. In particular, CD40–CD40L interactions are essential for some CD8⁺ T-cell responses but dispensable for others (103–105). The level of inflammation (i.e., the type of pathogen or vaccine), the amount of antigen, the precursor frequency of responding CD8⁺ T cells, and the route of infection all participate in determining the factors required to mount a successful CD8⁺ T-cell response. In general, the cellular, soluble factors, and costimulatory requirements for a CD8⁺ T-cell response are defined by their ability to cause robust expansion of responding CD8⁺ T cells. However, some factors may not participate via provision of a growth stimulus, but rather by augmenting, or dampening, cellular functions such as effector function or homing. Because of the concerted often simultaneous efforts of multiple cellular and soluble factors, it is oftentimes difficult to assign precise functions to one participant versus another. Additionally, as is often the case, one factor may affect the action of another by upregulation of necessary receptors or activation of a distinct cellular subset.

The original definition of CD4⁺ T-cell help for CD8⁺ T-cell responses centered on the production of IL-2 by CD4⁺ T cells (106). Later studies suggested that the way in which CD4⁺ T cells helped CD8⁺ T cells was through activation of APC and upregulation of CD40 (107–111). The initial activation of APCs would then allow naive CD8⁺ T cells to subsequently encounter activated APCs, resulting in priming and expansion. Thus, in this scheme of a temporal bridge between CD4⁺ and CD8⁺ T-cell activation, CD4-derived IL-2 did not appear to play a role in CD8⁺ T-cell activation. The reality of the situation no doubt contains elements of both models, and the interpretation of the original results may now be seen in the light of recent developments. For example, new findings indicate that rather than CD4⁺ cells expressing CD40L that interacts with CD40 on APCs, APCs can be induced by viral infection to express CD40L that then interacts with CD40 expressed by CD8⁺ T cells, even in the absence of CD4⁺ T cells (112). Thus, there are at least two pathways for driving CD8⁺ T-cell effector development: (i) CD4 dependent, in which CD4⁺ T cells may activate the DC but also will provide IL-2 to activated CD8⁺ T cells and (ii) CD4 independent, in which APCs activated via TLR or infection are able to directly drive CD8⁺ T-cell activation. The latter scenario may also require IL-2, perhaps through autocrine production. Indeed, our results from the analysis of the largely CD4⁺ T-cell independent CD8⁺ T-cell response to VSV infection demonstrate a partial reliance on IL-2 for optimal expansion and SLEC generation (86). By comparison, the CD4-dependent CD8⁺ T-cell response to intraperitoneal VV infection is IL-2 dependent but does not require CD40 (86). The latter result implies that in some situations, CD4⁺ T-cell help operates through provision of IL-2 rather than via APC activation. The route of infection may also affect the CD4⁺ T-cell dependence of the CD8⁺ T-cell response. Thus, the overall expansion of CD8⁺ T cells responding to intranasal VV infection is normal in the absence of CD4⁺ T cells (113). However, the resulting memory cells are functionally defective because of heightened programmed death-1 (PD-1) expression (113). Overall, the available results indicate that the mechanism of CD4⁺ T-cell help cannot yet be codified, and the precise factors involved in ‘help’ need be examined for each immunization condition.

The kinetics of the requirement for IL-2 during the CD8⁺ T-cell response is also instructive. IL-2 is not required for initiation of cell cycle in responding CD8⁺ T cells (114,115). Both autocrine and paracrine IL-2 effects have been observed. When IL-2-deficient CD8⁺ T cells are transferred into IL-2 competent hosts, the CD8⁺ T-cell response is actually greater than that mounted by IL-2-competent CD8⁺ T cells, particularly in non-lymphoid tissues. Moreover, IL-2 competent CD8⁺ T cells can ‘rescue’ IL-2-deficient T cells, demonstrating paracrine transfer of cytokine among ‘brothers’. These results suggest that IL-2 may play a negative role in dampening the response, but in these studies an analysis of the EEC/MPEC/SLEC subsets was not performed. Rather than being involved during early immune response initiation, IL-2 acts during the late primary response, but still prior to the peak of the expansion phase (Figs 2 and 3). At those later time points, IL-2 will serve to maximize CD8⁺ T-cell growth and promote survival (86,115). Furthermore, maximal IL-2-mediated growth activity correlates with maximum expression of IL-2R α (CD25), which is required for formation of the high-affinity IL-2 receptor. Of particular interest is the finding that CD4⁺ T cells augment CD25 expression (86). Whether this effect is through IL-2-mediated upregulation of CD25 is not yet known, although *in vitro* data support this possibility (116) and administration of IL-2 to helpless CD8⁺ T cells rescued CD25 expression (86).

In addition to enhancing CD8⁺ T-cell expansion and survival, both CD4⁺ T-cell help and IL-2 have also been implicated in imprinting CD8⁺ T cells during the primary response. In this scenario, CD4⁺ T cells and/or IL-2 affect the responding CD8⁺ T cells not through providing growth signals, but via programming of the CD8⁺ T cells to allow the resultant memory cells to respond to secondary antigenic challenge (113,117–120). However, later work suggests that CD4⁺ T cells are important not for programming but for maintenance of CD8⁺ T-cell memory after the expansion phase by an as yet undefined mechanism (121). Other studies have variously

shown depending on the immunogen or pathogen employed that CD4⁺ T cells are neither essential for primary nor recall responses, only needed in the primary response, or only needed in the secondary response (122–126). Similarly, IL-2 apparently ‘programs’ primary LCMV or LM-specific CD8⁺ T cells to generate competent memory cells (120) or is required as a growth factor in both the primary and secondary response to acute LCMV infection (127). The reason for the discrepancies between the different studies is not clear, as at least in some cases the response to the same pathogen was being analyzed. In our studies, we examined the role of CD4⁺ T-cell help and IL-2 in the development of the three main CD8⁺ T-cell effector subsets: EECs, MPECs, and SLECs. The primary CD8⁺ T-cell response to infection with VV intraperitoneally or LM is CD4⁺ T-cell dependent, while the VSV CD8⁺ T-cell response is CD4⁺ T-cell independent. In the absence of CD4⁺ T cells, early expansion of LM or VV-specific CD8⁺ T cells is normal, but continued expansion does not occur leading to development of a reduced memory pool. In CD25^{-/-} CD8⁺ T cells, the major defect observed during the effector phase is the development and/or survival of SLECs. Concomitant increases in the proportion of EECs and MPECs are also evident in CD25^{-/-} effector CD8⁺ T cells. Interestingly, although based on overall expansion the CD8⁺ T-cell response to VSV infection is CD4⁺ T-cell independent, SLEC generation is partially defective when CD4⁺ T cells or IL-2R α signaling is absent during the VSV infection. The effect on the overall response is likely not as dramatic as observed with LM infection, because of the fact that many fewer SLECs are generated in response to VSV versus LM infection (J.J. Obar and L. Lefrançois, unpublished observation). In addition, during all three infections (VSV, LM, and VV), CD4⁺ T cells are required for maximal induction of CD25 expression by responding CD8⁺ T cells (86). Thus, the degree of dependence of the total CD8⁺ T-cell response on CD4⁺ T cells and IL-2 is linked, at least in part, to the relative proportions of EECs, MPECs, and SLECs that are produced in response to different infections or immunogens. Considering that ‘helpless’ or CD25-deficient memory CD8⁺ T cells are reportedly defective, we had predicted that MPEC would be preferentially disturbed by the absence of CD4⁺ T cells or IL-2R α signaling. Yet, SLECs are selectively lost in these situations. Moreover, in CD4⁺ T-cell-depleted mice or in intact mixed bone marrow chimeras produced with CD25^{-/-} and wildtype BM, both helpless and CD25^{-/-} memory CD8⁺ T cells undergo a robust expansion upon challenge that is equal to or greater than the response of wildtype memory CD8⁺ T cells (86,123). However, as in the primary response, SLEC generation is blunted in the recall response of CD25^{-/-} CD8⁺ T cells.

Our hypothesis then is that CD4⁺ and CD8⁺ T-cell activation occur essentially simultaneously. In certain infections, initial APC activation is mediated via TLR signaling or other pathogen-associated molecular pattern-sensing mechanisms. As CD4⁺ T-cell expansion is believed to be considerably less robust than CD8⁺ T-cell expansion (128,129), CD4⁺ T-cell numbers will lag behind CD8⁺ T-cell numbers during the early phases of the response. Once a critical mass of CD4⁺ T cells is available, then sufficient IL-2 is produced to provide help to the responding CD8⁺ T cells via CD25 upregulation, leading to enhancement of proliferation (Fig. 3). In other cases, such as herpes simplex virus I infection, the CD8⁺ T-cell response requires ‘licensing’ of DCs by CD4⁺ T cells in the first couple days after infection (125), although CD4⁺ T-cell-derived IL-2 may also play a role in CD8⁺ T-cell expansion.

Memory CD8⁺ T cells and cytotoxicity

The passage of naive CD8⁺ T cells through an effector stage results in permanent genetic, phenotypic, and functional changes that can be detected in the memory CD8⁺ T-cell population (36,37,130–132). One of the functions that is retained by a subset of memory cells is the ability to mediate direct *ex vivo* cytotoxic activity (36,37). Those cells that retain direct *ex vivo* cytotoxic activity are termed T_{EM} cells and are preferentially localized to non-lymphoid tissues, such as the lung and intestinal mucosa (37,133). Additionally, in the mouse spleen a population of T_{EM} cells and a non-lytic T_{CM} cell population are found, both of which can convert to

cytotoxic memory cells upon entry into non-lymphoid tissues (134). With secondary and tertiary boosts, the T_{EM} cell population progressively increases in lymphoid and non-lymphoid tissues (133). Nearly all tertiary memory CD8⁺ T cells, except those in the LN, express granzyme B (133). These findings reinforce the linear differentiation model as retention of lytic activity by memory cells is unlikely to have occurred without passage through an effector stage in the primary response. We have also examined the effector subsets and their protective abilities in secondary and tertiary CD8⁺ T-cell responses to infection with VSV and LM. Early after a secondary challenge, the majority of the effector cells are made up of KLRG1^{high} cells, but a substantial portion of these also express IL-7R α double-positive effector cell (DPEC) (Joshua J. Obar and Leo Lefrançois, unpublished data). After several months the memory population was comprised of roughly equal proportions of SLEC, DPEC, and MPEC phenotype subsets. Given these findings, the moniker of ‘short-lived’ may not apply in secondary responses. Nonetheless, challenge of each of the memory subsets revealed a hierarchy of responsiveness in terms of the ability to mount a proliferative response: MPEC > DPEC > SLEC. The ability to respond via proliferation also correlated to the protective capacity of each subset. Thus, KLRG1 expression correlated with reduced proliferative and protective abilities, further supporting the concept that KLRG1 may be mediating a negative regulatory effect on the response.

Onward through the fog

As discussed here, the fog is beginning to lift on the mysteries surrounding memory T-cell development. Several of the factors regulating effector CD8⁺ T-cell heterogeneity have been identified, and this knowledge has set the stage to delve deeper into the control of the effector phase and the ensuing development of memory CD8⁺ T-cell subsets. Molecular and genetic analyses of the earliest effector cell populations are likely to reveal considerable heterogeneity. Studying the causal effects of this heterogeneity will eventually allow us to identify the very earliest events required for effector subset specification. This analysis will in turn provide the means to determine the precise pathway from effector ‘stem cells’ to memory CD8⁺ T-cell generation.

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Fig. 1. Granzyme B is expressed by all effector CD8⁺ T-cell subsets

C57BL/6 mice were infected i.v. with either 10^3 colony-forming unit Lm-ova or 10^5 PFU vesicular stomatitis virus-ova. Five days later, Ova/K^b-specific CD8⁺ T cells in the spleen were phenotyped using the cell surface molecules KLRG1 and CD127 to identify the early effector cells (EECs), short-lived effector cells (SLECs), and memory precursor effector cells (MPEC) populations. Intracellular granzyme B levels were then examined within each population and compared with naive CD8⁺ T cells (CD11a^{low} CD44^{low}). Granzyme B levels for naive CD8⁺ T cells are displayed in the filled gray histogram, while the granzyme B levels in the Ova/K^b-specific effector CD8⁺ T-cell populations are in the open histograms: EECs (red line), SLECs (black line), and MPECs (blue line).

Fig. 2. Linear differentiation model of effector and memory CD8⁺ T cells

Early activation of the naive CD8⁺ T-cell population results in the generation of the early effector cell (EEC) population, which begins differentiation into short-lived effector cells (SLEC) and memory precursor effector cells (MPECs). These early differentiation events (i.e., the first 3–4 days) correspond to times when high levels of interleukin (IL)-12, IL-15, and interferon- α - β are found. The next cytokine that appears to work on the responding CD8⁺ T-cell population is IL-2, as the high-affinity IL-2 receptor is only expressed transiently during this time period (i.e., days 3–5). IL-2 not only works to amplify the whole CD8⁺ T-cell population but also enhances the differentiation of the EEC population to SLECs. Around the peak of the CD8⁺ T-cell response, high levels of transforming growth factor (TGF)- β can be found. TGF β will oppose the actions of homeostatic levels of IL-15 and promote the preferential apoptosis of the EEC and SLEC populations leaving only the MPEC population. The levels of IL-15 in the tissues will also regulate this process as SLECs are exquisitely dependent on IL-15 for their survival, whereas the MPEC population is more dependent on IL-15 for long-term homeostatic proliferation and is dependent on IL-7 for survival.

Fig. 3. Model for the role of interleukin (IL)-2 production from ‘helper’ CD4⁺ T cells in effector CD8⁺ T-cell differentiation and expansion

Activation of both CD4⁺ and CD8⁺ T cells by dendritic cells will occur simultaneously in lymphoid organs. For both CD4⁺ and CD8⁺ T cells, activation and subsequent proliferation is mediated by T-cell receptor:pMHC (peptide: major histocompatibility complex) interactions and costimulatory molecules, such as CD28:CD80/86. When the ‘helper’ CD4⁺ T-cell population reaches a critical mass enough paracrine IL-2 will be produced to ‘help’ maintain the expansion and differentiation of CD8⁺ T-cell responders. IL-2 will act on the CD25⁺ early effector cells (EECs) population and further upregulate the expression of CD25, at which time it will help direct the differentiation of the EEC. IL-2 will promote the preferential differentiation toward short-lived effector cells, but is likely important in the expansion of all effector cells.

Table 1

Factors regulating effector cell heterogeneity

	Outcome	Reference(s)
Cytokines		
IL-2	SLEC differentiation Effector cell expansion	(86)
IL-12	SLEC differentiation	(57)
	Effector cell expansion	(75,76)
IL-15	SLEC survival	(57,89)
	Memory cell turnover	(102,135)
TGF β	SLEC apoptosis	(59)
IFN α/β	SLEC differentiation Effector cell survival	Joshua J. Obar and Leo Lefrançois, unpublished results (69,70)
IFN γ	SLEC differentiation	(74)
Transcription factors		
T-bet	SLEC differentiation	(57)
Blimp1	SLEC differentiation	(84,85)
Xbp-1	SLEC formation	(136)
Gfi-1	Represses IL-7 α	(137)
GABP α	Enhances IL-7 α	(137)
Other		
OX40	Promotes MPEC formation	(138)
CD4 'help'	SLEC differentiation	(86)
Adjuvants or drugs		
IL-2/ α IL-2 complex	Enhances SLEC accumulation	(89)
IL-7/ α IL-7 complex	Enhances MPEC accumulation	(89)
IL-15/IL-15 α complex	Enhances SLEC accumulation	(89)
Rapamycin	Enhances MPEC formation	(82)
Metformin	Enhances memory formation	(83)
CpG ODN	Enhances SLEC formation	(57,74,80)

IL, interleukin; SLEC, short-lived effector cell; TGF, transforming growth factor; IFN, interferon; MPEC, memory precursor effector cell.