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The rationale for the IL-2-independent generation of the self-renewing central memory $CD8^+$ T cells

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Summary: Clones of $CD8^+$ T cells that have been selected in the primary response must have a mechanism by which they can continuously or intermittently generate new effector cells. Several years ago, this mechanism was proposed to involve a self-renewing, stem cell-like subset that could avoid the differentiating effects of interleukin-2 (IL-2). The model considered the stem cell subset to be contained within the central memory population of $CD8^+$ T cells (T_{CM}). This proposal was inconsistent with subsequent findings suggesting that all antigen-activated $CD8^+$ T cells differentiated to effector cells (T_{EFF}) during the primary response and that T_{CM} developed during the memory phase by de-differentiating from effector memory cells (T_{EM}). However, findings have since been reported that support the stem cell model. First, studies indicate that T_{EM} do not serve as the precursors of T_{CM}. Second, transcriptional repressors of IL-2 signaling do enhance the memory response. Third, memory cells lacking effector functions and with a capacity to replicate in a secondary response develop in the absence of signaling through the IL-2/IL-15 receptor. Taken together, these findings suggest that antigen-activated $CD8^+$ T cells with a stem cell-like capability for maintaining proliferative potential develop by an unknown IL-2-independent process. The challenge is now to identify this unknown pathway of clonal expansion.

Introduction

The biological purpose of the antigen-dependent phase of T-cell development is to generate numbers of cells with differentiated effector functions that are sufficient to resolve or control an infection. This function is especially challenging. Within the pool of naïve T cells, the frequency of clones that are specific for individual microbial antigens is low, and, at least for $CD8^+$ T cells, the number of T_{EFF} that are needed to control infections is high, so that each clone must generate perhaps as many as 10^5 T_{EFF} in 7–10 days. Moreover, if the primary response controls but does not eliminate the infectious agent, continuous, uninterrupted production of T_{EFF} may be required. Even if the host eliminates the

infectious agent during the primary response, it may persist in the environment and possibly cause a secondary infection, requiring the production of new T_{EFF}. Therefore, CD8$^+$ T-cell clones that have been selected in the primary response must have the potential for generating T_{EFF} continuously or intermittently for the individual's lifetime. To do so, antigen-selected CD8$^+$ T cells should have a self-renewing, immortal, stem cell-like stage of development. A stem cell stage during antigen-triggered CD8$^+$ T-cell development was proposed in 2001 (1), and here we summarize experimental findings bearing on this proposal that have since been published.

The most appropriate experimental system in which to study the possible role of self-renewal in the continuous generation of CD8$^+$ T_{EFF} would be a persistent viral infection. Although there have been studies of the CD8$^+$ T-cell response in persistent viral infections in the mouse, these have either emphasized the ability of the infection to overwhelm the CD8$^+$ T-cell response, as occurs with clone 13 of lymphocytic choriomeningitis virus (LCMV) (2) that impairs the antigen-presenting functions of dendritic cells (3), or have shown continued expansion of epitope-specific CD8$^+$ T cells, as in murine cytomegalovirus (mCMV) infection (4), but have not yet evaluated the developmental basis of the continuing CD8$^+$ T-cell response. Instead, the question of how antigen-stimulated CD8$^+$ T cells maintain their proliferative potential has been studied mainly in relation to the development of memory CD8$^+$ T cells that mediate the replicative component of a secondary response. Therefore, review of work that pertains to a self-renewing stage of antigen-stimulated CD8$^+$ T-cell differentiation must be done in the context of intermittent rather than a continuous stimuli for clonal expansion, and it must be assumed that the memory CD8$^+$ T-cell that generates the burst of new effector cells in a secondary response is closely related to the proposed stem cell that maintains the production of new T_{EFF} during persistent viral infections. This emphasis on the proliferation of memory CD8$^+$ T cells also accounts for the absence in this review of a discussion of the role of memory CD8$^+$ T cells that have immediate effector function to provide early defense against secondary infections.

Memory CD8$^+$ T cells are comprised of central memory and effector memory subsets

In 1999, Lanzavecchia and colleagues (5) discovered that assessing human memory CD4$^+$ and CD8$^+$ T cells for their expression of the chemokine receptor CCR7 revealed the presence of two functionally distinct subsets: CCR7$^+$ memory T cells that tended to resemble naïve T cells in being CCR7$^+$ and in lacking effector functions, and CCR7$^-$ memory T cells that were capable of immediate effector activities. They named the CCR7$^+$ memory T cells T_{CM}, to acknowledge their potential for homing to secondary lymphoid organs, and termed the CCR7$^-$ memory T cells T_{EM}, because of their relatively differentiated status. T_{CM} were considered to be responsible for the generation of new T_{EFF} during secondary responses, and T_{EM} to mediate rapid host defense while new T_{EFF} were being produced. The correspondence between a memory cell's potential for homing to lymphoid or non-lymphoid tissues, the absence or presence of potential effector function, and a cellular division of labor with respect to the two tasks of a secondary response, proliferation and immediate effector function, was so intuitively appropriate that immunologists quickly adopted this nomenclature.

The occurrence of the T_{CM} and T_{EM} subsets of memory cells now underpins most analyses of the function and development of antigen-stimulated T cells. T_{CM} not only express CCR7 but also high levels of CD62L, enabling them to recirculate between the blood and secondary lymphoid organs, while T_{EM}, by definition being CCR7$^-$ and CD62L^{low}, tend to be found primarily in peripheral tissues and the non-lymphoid zones of the spleen (6–8). Lymphoid tissue is adapted for supporting antigen-dependent, secondary proliferative responses of memory T cells leading to the generation of new T_{EFF}, while peripheral, non-lymphoid tissue does not usually have this capability and represents potential areas of infection, where it would be appropriate for memory T cells to exercise immediate effector function. Although mice deficient in lymphotoxin-α and lacking organized secondary lymphoid tissue have a primary response to pulmonary influenza infection (9), this does not necessarily indicate that a lymphoid architecture is not required for antigen-induced CD8$^+$ T-cell expansion, as the response is both delayed and diminished in these mice, and it may reflect the function of bronchoalveolar lymphoid tissue (10). The proposed differing capabilities for replication in the T_{CM} and T_{EM} CD8$^+$ T-cell subsets also acknowledges the possibility that development of effector functions might be associated with loss of replicative function, as usually occurs with effector differentiation in other cellular lineages. If so, then the possibility that T_{EM} and T_{EFF} might be depleted in the early phases in a secondary infection provides the rationale for the occurrence of the T_{CM} subset, which retain the proliferative capability of the naïve CD8$^+$ T cells. Therefore, the proposal that memory T cells could be divided into these two subsets was insightful

and constructive, in that it defined new experimental questions for understanding the function and development of memory CD8$^+$ T cells. These questions included the following. Is it correct that T_{CM} accounts for the replicative component of the secondary response? What are the characteristics of T_{CM} that allow this subset to retain replicative function? What are the developmental relationships between naïve CD8$^+$ T cells, T_{CM}, T_{EM}, and T_{EFF}?

Models for the development of central and effector memory CD8$^+$ T cells

Three general models have been proposed for the developmental pathways leading from naïve CD8$^+$ T cells to T_{CM}, T_{EM}, and T_{EFF} (*Fig. 1*). These models are presented in this section, and the experimental evidence for them is in the next section. Lanzavecchia and Sallusto (11) proposed in 2000 the 'progressive-differentiation model', which puts forward the concept that naïve T cells progress through 'hierarchical thresholds' for proliferation and differentiation as the strength and duration of interaction with dendritic cells and cytokines is increased. T cells receiving the weakest signals are 'unfit' and do not survive. The next threshold of signaling generates the precursors of T_{CM} that are fit and survive but have not acquired effector functions. Even more signaling leads to acquisition of effector functions, but not loss of essential long-term survival characteristics, such as expression of CD127 [interleukin-7 receptor-α (IL-7Rα)]. The highest level of signaling causes development of terminally differentiated T_{EFF} that cannot survive into the memory phase. These different levels of signaling are a consequence of stochastic interactions between T cells and dendritic cells, which may reflect not only the level of activation of the antigen-presenting dendritic cells but also competition among proliferating T cells for interaction with dendritic cells. After clearance of antigen, the precursors of T_{CM}, being the least differentiated of the antigen-experienced memory cells and maintaining the proliferative potential and homing receptors of naïve cells, become resting T_{CM} and populate secondary lymphoid tissues, where they are poised to mount secondary proliferative responses. T_{EM}, having progressed to an effector stage of development and having changed their chemokine receptors because they had received stronger signals during the primary response than did T_{CM}, populate peripheral non-lymphoid tissues. The most stimulated T_{EFF} have lost their capacity to respond to survival and homeostatic cytokines and do not persist into the memory phase.

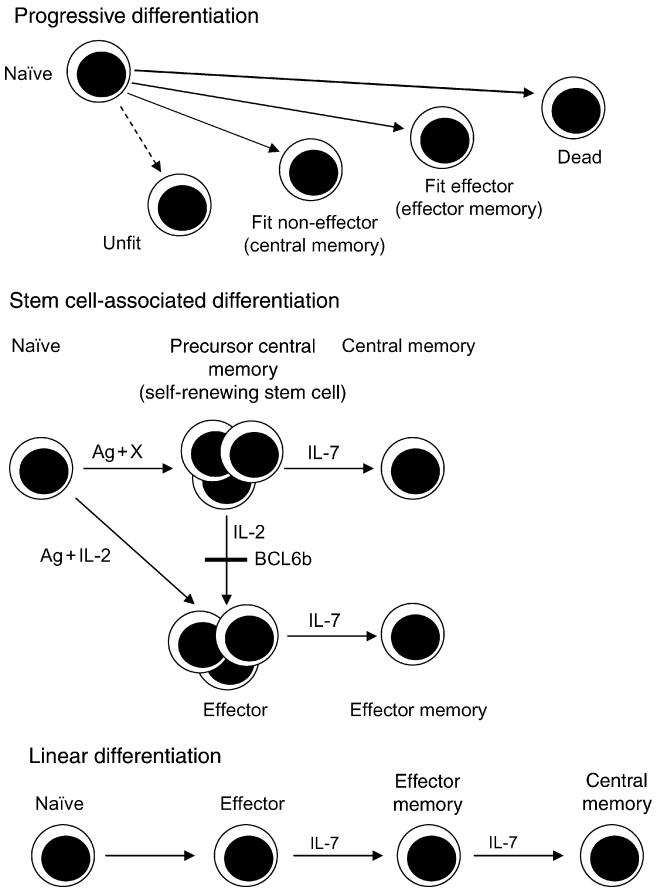

***Fig. 1.* Three models for the development of central memory and effector memory CD8$^+$ T cells during the primary immune response.** Shown are the progressive-differentiation model (11), the stem cell-associated differentiation model (1), and the linear differentiation model (13). In the stem cell-associated model of differentiation, X refers to the unknown agonist that causes IL-2/IL-15-independent proliferation leading to the development of the precursor central memory cell.

The critical elements of this model are that there is a hierarchy of irreversible differentiation, proceeding from the naïve T cell → T_{CM} → T_{EM} → T_{EFF}. The T_{CM} stage, being the least differentiated of the antigen-stimulated T cells, retains essentially all of the developmental options of a naïve cell, including its capacity for marked clonal expansion. Also, the developmental process is governed by the relative availability of a common set of signals and not by specific signals for each stage, and for this reason, it is appropriately characterized by its proponents as a stochastic developmental process.

In 2001, a second proposal was considered that might be termed the 'stem cell model' (1). It resembles the progressive differentiation model in believing the differentiation process to be irreversible, but it differs in that the T_{CM} subset is considered to be a self-renewing, stem cell-like stage of development that enables it to serve as an inexhaustible source of T_{EFF} for both chronic and intermittent infections. In the sense that this subset is proposed also to provide the precursors of

effector cells in primary and persistent infections, not only for secondary responses, the T_{CM} designation is not quite appropriate. In persistent infections, these replicating T cells (pre-T_{CM}?) do not require one of the cardinal properties of a memory cell, which is long-term survival in the absence of antigen. However, the known ability of stem cells in other organ systems to adjust the rate at which they replicate according to the relative need for cells with differentiated functions encompasses both continuous and intermittent generation of effector cells by T_{CM}. A self-renewal function is implied in the progressive differentiation model with 'weak' signals inducing replication without differentiation. However, in the stem cell model of antigen-dependent $CD8^+$ T-cell differentiation, the T_{CM} stage is the result of either unique proliferation signals that do not cause differentiation or an active process that prevents effector differentiation of the antigen-stimulated cells. This stem cell model believes that the high number of cell cycles that T_{CM} go high through to account for their clonal expansion during a primary response high indicates that they have received ample signaling, and not the weak signal proposed by the progressive differentiation model. The biological problem that the existence of the T_{CM} subset presents is how such extensive cellular replication can be uncoupled from effector differentiation. The only known means for $CD8^+$ T-cell replication, stimulation by IL-2 or IL-15, if repetitively applied, causes effector differentiation (12). The stem cell hypothesis proposed that a mechanism analogous to that of BCL-6, a transcriptional repressor of the germinal center B cell that inhibits IL-2-induced differentiation to a plasma cell, has a role in arresting the differentiation of the developing T_{CM}.

The third model was proposed in 2003, termed the 'linear differentiation model' (13). This model differs from the two previous models in putting forward the concept that T_{EFF} develop directly from naïve $CD8^+$ T cells, T_{EM} from T_{EFF} after the primary infection is resolved, and then T_{CM} only during the memory phase by a gradual process of de-differentiation from the T_{EM} subset. The only signal that has been considered to be required for the conversion of T_{EM} to T_{CM} is IL-7 (14), but this cytokine probably functions only as a survival factor (15) and not to regulate differentiation. One of the most interesting implications of this model is that it does not provide a mechanism for the ability of $CD8^+$ T cells to cope with persistent infections. If T_{CM} is the subset that has secondary proliferative function and if the generation of new T_{EFF} during a persistent infection is functionally analogous to this process in a secondary response, the proposal that a 'rest period' is required for the development of the T_{CM} subset means that $CD8^+$ T cells would not be able to generate new T_{EFF} during persistent viral infections. Despite this aspect of the model not being in accord with many examples of the $CD8^+$ T-cell response coping with persistent viral infections, with perhaps the most striking example being the continuous production for years of $CD8^+$ T_{EFF} in patients infected with human immunodeficiency virus until virally induced severe depletion of $CD4^+$ T cells occurs, the experimental findings in the study presenting the linear differentiation model were compelling. This model was widely accepted as the mechanism for the development of memory $CD8^+$ T cells (16).

Experimental analyses of development models

In the original description of the $CD8^+$ T_{CM} and T_{EM} subsets, the $CCR7^+CD8^+$ T_{CM} subset had no effector functions, including an absence of interferon-γ (IFN-γ) production (5). Most studies of murine $CD8^+$ T-cell differentiation have used high expression of CD62L as the phenotypic marker of the T_{CM} subset, and they have occasionally found cells with effector function in the $CD62L^{high}$ memory population. Although this finding would apparently argue against a clear association between distinct tissue homing functions and immediate effector capabilities, it is more likely that $CD62L^{high}$ memory cells with effector functions indicate an asynchronous differentiation process, making homing receptor expression an imperfect marker of differentiation. For the sake of consistency with the original description of these cells, when T_{CM} are referred to in the review, the definition remains that of Lanzavecchia and colleagues (5), this being a central homing memory cell without immediate effector function.

The primary study forming the basis of the linear differentiation model set the agenda for subsequent investigators by showing that T_{CM}, defined as $CD62L^{high}$ memory $CD8^+$ T cells taken at least one month after the resolution of a primary infection, mediated a more effective secondary response when adoptively transferred to recipient mice than did $CD62L^{low}$ T_{EM} taken at the same time (13). The efficacy of the transferred cells was measured by viral clearance from several different sites of challenge and by expansion and generation of new effector cells. The development of $CD62L^{high}$ memory cells appeared to be from $CD62L^{low}$ cells, because very few of the former were found during or immediately after the primary response, and during the memory phase the proportion of memory cells that were $CD62L^{high}$ increased. Furthermore, 25 days after adoptive transfer of an almost homogenous population of $CD62L^{low}$ memory cells, 45% of the transferred cells were $CD62L^{high}$. These findings were interpreted as indicating that secondary replicative responses were mediated

by T_{CM} and that T_{CM} were not present during the primary response but slowly developed from T_{EM} during the memory phase. However, additional findings suggested that selective expansion of the $CD62L^{high}$ cells also contributed to the shift in the phenotype of the memory cells. $CD62L^{high}$ memory cells were shown to replicate more rapidly than $CD62L^{low}$ cells, whether measured in non-lymphopenic or lymphopenic environments by dilution of carboxyfluorescein diacetate succinimidyl ester (CFSE) or by uptake of bromodeoxyuridine (BrdU). Thus, the T_{CM} mediating effective secondary responses may have been present in small numbers early in the memory phase as $CD62L^{high}$ cells, but they required time for homeostatic expansion to become the dominant memory population. Interestingly, this report also showed that T_{CM}, in addition to expressing a naïve cell's level of CD62L, had another characteristic of a less differentiated cell, which was the ability to produce IL-2. Therefore, the 'linear differentiation pathway' is truly a de-differentiation pathway. Although de-differentiation has been reported to occur in plasma cells in which BCL-6 has been introduced (17), the phenomenon is sufficiently rare in biological systems to merit publication of its experimental demonstration. Even though an inability to account for the capacity of $CD8^+$ T cells to cope with persistent viral infections and the need to posit a process of de-differentiation weaken the case for the linear progression model, it is important to recognize that the study on which this model is based is the first to demonstrate that the T_{CM} subset is responsible for secondary proliferation.

Five studies using adoptive transfer of memory cells have now tested aspects of this model (*Table 1*). Only one has not found that replicative memory responses are mediated by the T_{CM} subset. Roberts and Woodland (18) demonstrated that $CD62L^{low}$ memory $CD8^+$ T cells elicited by primary infection with Sendai virus were more effective than $CD62L^{high}$ cells in generating new effector cells after adoptive transfer to recipient mice and secondary challenge with intranasal Sendai virus. However, a second report by this group one year later (19) modified this conclusion by showing that the relative capabilities of the T_{EM} and T_{CM} subsets were dependent on the time at which they were assayed for this function. When taken one month post-primary infection, $CD62L^{low}$ memory cells were more effective, whereas at 13 months post-primary infection, the $CD62L^{high}$ T_{CM} subset was more effective in a secondary infection with Sendai virus. Although this observation could reflect a more rapid acquisition of replicative function than of high CD62L expression by the memory cells destined to become T_{CM}, the first report by this group may be the only instance in which cells identified as T_{EM} by their relative expression of CD62L were more effective in generating new T_{EFF} in a secondary response than T_{CM}. The authors suggested that mucosal infections, such as that caused by Sendai virus, may alter the memory response as compared to infections at other sites, a possibility that needs to be investigated further, given the sometimes different strategies adopted by the immune system to cope with mucosal infections.

In contrast to the analysis by Roberts and Woodland (18), that by Bachmann and colleagues (20), which involved infection of mice with LCMV, confirms many of the findings of Wherry *et al.* (13), the most notable being that the $CD127^+CD62L^{high}$ T_{CM} subset of memory cells was most

***Table 1.* Central memory versus effector memory $CD8^+$ T cells and secondary replicative responses**

Study	Immunological challenge	Memory subset transferred	2° proliferation	Conversion T_{EM} to T_{CM}	2° proliferation of 'converted' cells
Wherry *et al.* (13)	LCMV, Vaccinia/gp33	1–2 mon. $CD62L^{high}$ 1–2 mon. $CD62L^{low}$	Good Poor	Yes	Not assessed
Roberts and Woodland (18)	Sendai	$CD62L^{high}$ $CD62L^{low}$	Less effective More effective	Not assessed	Not assessed
Bachmann *et al.* (20)*	LCMV, Vaccinia/gp33	$CD127^+/CD62L^{high}$ $CD127^+/CD62L^{low}$	Good Poor	Yes	Not assessed
Bouneaud *et al.* (21)	Male bone marrow cells	>1.5 mon. $CD62L^+$ >1.5 mon. $CD62L^-$	Good Poor	Yes	2/7 mice responded with 47% of cells converted
Marzo *et al.* (23)†	LCMV	$CD62L^{high}$ $CD62L^{low}$	Good Poor	No	Not applicable
Roberts *et al.* (19)	Sendai	12 mon $CD62L^{high}$ 12 mon $CD62L^{low}$	More effective Less effective	Not assessed	Not assessed

*In the Bachmann study, adoptively transferred gp33-specific TCR transgenic $CD8^+$ T cells were taken 10 days after LCMV infection, and subsets were re-transferred to naïve recipients that were then challenged with LCMV or vaccinia expressing gp33. Thus, these cells were not memory cells but were acutely responding cells, demonstrating the replicative potential of cells with a T_{CM} phenotype at the peak of a primary response.
†In the Marzo study, conversion of T_{EM} to T_{CM} was observed only when T_{EM} were derived from a large number of naïve cells.

effective in secondary replicative responses and that this subset had a greater capacity for the production of IL-2 than that of T_{EM} or T_{EFF} (20). These investigators also found that after adoptive transfer, CD127$^-$CD62L^{low} cells gradually reverted to a CD127$^+$CD62L^{high} phenotype, and CD127$^+$CD62L^{low} memory cells became CD127$^+$CD62L^{high}. However, the apparent conversions between subsets may have represented expansions of contaminating CD62L^{high} memory cells of greater homeostatic proliferative function, because it was noted that whereas recovery of CD127$^+$CD62L^{high} cells was 'almost quantitative', that of the CD127$^-$CD62L^{low} subset was poor. This study had an additional finding that may relate to the time at which T_{CM} develop. Adoptive transfer of LCMV-specific, CD127$^+$CD62L$^+$CD8$^+$ T cells obtained on day 10 or 15 (in the text it is written day 10, and in the legend for the figure describing this experiment it is written day 15) of a primary response provided naïve recipients with a capacity for a replicative secondary response to viral infection initiated one day after transfer. Therefore, in contrast to the linear differentiation model stating that T_{CM} develop slowly over a period of weeks during the memory phase, this finding suggests that they are present during the primary response (day 10) or very early in the memory phase (day 15). Even if it is argued that the cells that transferred the capability for a secondary response cannot be considered as memory cells because they were taken during or at the end of the primary response, they must be considered at least as 'pre-T_{CM}', which indicates that the commitment for T_{CM} development can occur earlier than the memory phase.

The possibility that an early development of T_{CM} may occur is also suggested by a study of the development of T_{CM} *in vivo* by using a non-viral system of administering male bone marrow cells to female mice to which had been transferred T-cell receptor (TCR) transgenic naïve CD8$^+$ T cells specific for the H-Y antigen Smcy3 (21). A number of findings were made in this complex study, but two of them are most relevant in the context of this review. First, the CD62L^{high} T_{CM} subset, not the CD62L^{low} T_{EM} subset, transferred replicative secondary responses, and, second, those memory cells that had converted from CD62L^{low} to CD62L^{high} during the memory phase were impaired in their ability to replicate when secondarily challenged. That is, the cells apparently belonging to the T_{CM} subset that developed during the memory phase, which the linear differentiation model presents as the subset mediating a secondary proliferative response, did not share with the T_{CM} developing earlier in the immune response the capacity for vigorous secondary proliferation. This finding did not precisely contradict the observations of Wherry *et al.* (13), which had assessed the secondary replicative function of all CD62L^{high} T_{CM} cells taken during the memory phase and not only the cells that had converted to the CD62L^{high} state during this period. Since a CD62L^{high} phenotype mediates homing to secondary lymphoid tissues, this observation also suggests that although the lymphoid environment may be necessary for a full proliferative recall response of a memory cell, it is not sufficient. Thus, the correlation noted between homing of *in vitro* activated, adoptively transferred CD8$^+$ T cells to secondary lymphoid tissue and non-lymphoid sites and the presence or absence, respectively, of antigen-stimulated proliferative function (22) does not explain fully the basis for proliferative function of the T_{CM} subset. The T_{CM} apparently must have two attributes that enable it to have a replicative secondary response, lymphoid homing capability and intrinsic proliferative potential. Therefore, these reports (20, 21), while validating the importance of the T_{CM} for secondary replicative responses, began to question whether the T_{CM} subset develops from the T_{EM} subset.

The most recent report relevant to this analysis of the development of the memory CD8$^+$ T-cell subsets presented evidence that this conversion is not physiological but is caused by the adoptive transfer of numbers of TCR transgenic naïve T cells that exceed the expected precursor frequency of naturally occurring naïve cells (23). CD62L^{low} memory CD8$^+$ T cells derived from a larger than normal number of adoptively transferred naïve cells converted to a CD62L^{high} phenotype during the memory phase of the response, whereas conversion was not observed with CD62L^{low} memory cells that had been generated from a low number of naive cells. It was proposed that the presence of large numbers of naïve cells leads to competition for limiting activation signals and the development of a 'transitional T_{EM}' subset that, because of the limiting activation signals, had not completed differentiation to a T_{EM} stage. The transitional T_{EM} is therefore 'unstable' and reverts to the CD62L^{high} phenotype, and it may be related to a recently described CCR7$^+$CD62L^{low} phenotype of responding, adoptively transferred, TCR transgenic CD8$^+$ T cells (24). Overall, this report argues against the linear differentiation model, which is based on experiments using adoptively transferred TCR transgenic T cells, and supports the progressive differentiation model that predicts that limiting activation signals are associated with the development of transitional cells that are intermediate between two stages of development.

In summary, studies of the behavior of adoptively transferred memory CD8$^+$ T cells have almost always found that the CD62L^{high} T_{CM} subset is principally responsible for the proliferative component of the secondary response. The

frequent finding of CD62L^{low} memory cells converting to a CD62L^{high} state during the memory phase has been recently correlated with a high precursor frequency of antigen-specific naïve cells, perhaps indicating that the T_{EM} to T_{CM} conversion is an artifact of the adoptive transfer model. However, secondary responses, which are initiated with a high precursor frequency of antigen responsive T_{CM}, might conceivably generate the 'transitional' T_{EM} subset. Even if there are circumstances in which the T_{EM} to T_{CM} conversion might normally occur, the CD8$^+$ T cells that become CD62L^{high} during the memory phase apparently do not have the proliferative function of the T_{CM} subset in a secondary response. This observation has another potential meaning, which is the likelihood of heterogeneity within the CD62L^{high} population of memory CD8$^+$ T cells, with some cells having this phenotype not being able to mediate a secondary replicative response. These findings strongly suggest that a model in which T_{CM} develop from T_{EM} during the memory phase does not apply to most circumstances of antigen-dependent CD8$^+$ T-cell development. T_{CM} must therefore develop during the primary response, and the report by Bachmann *et al.* (20) provides direct evidence in favor of this conclusion. In the absence of the conversion of T_{EM} to T_{CM} during the memory phase, as shown by the study of Marzo *et al.* (23), the improvement with time of replicative memory must reflect changes within the T_{CM} subset itself. This may be explained by the greater homeostatic expansion of the T_{CM} subset than within the T_{EM} subset, leading to an absolute increase in the number of T_{CM}.

These adoptive transfer studies identifying T_{CM} as the subset that generates T_{EFF} in a secondary response also imply but do not clearly show that T_{CM} precede T_{EM} in development and are therefore less differentiated than T_{EM}. Although Bouneaud *et al.* (21) and Marzo *et al.* (23) suggest that all three products of a primary response, T_{CM}, T_{EM}, and T_{EFF}, develop independently, the ability of the T_{CM} subset to give rise also to T_{CM}, T_{EM}, and T_{EFF} (24) during a secondary response suggests that pre-T_{CM} could also serve as the source of T_{EM} and T_{EFF} during a primary response. This function for pre-T_{CM}, which remains to be demonstrated, would be most consistent with the stem cell model of development.

With regard to this question of whether CD8$^+$ T_{CM} are less differentiated than T_{EM}, in the original description of T_{CM} and T_{EM}, CD4$^+$ T_{CM} could produce IL-2 but not IFN-γ or IL-4, the effector cytokines marking T-helper 1 (Th1) and Th2 development, whereas CD4$^+$ T_{EM} did produce these effector cytokines (5). This finding was taken as evidence that CD4$^+$ T_{CM} were less differentiated than CD4$^+$ T_{EM}. It is curious, then, that the relatively greater capacity of CD8$^+$ T_{CM} than T_{EM} to produce IL-2 is not necessarily considered as indicating that CD8$^+$ T_{CM} are less differentiated than the CD8$^+$ T_{EM} that can make IFN- γ. Even though IL-2 has no antiviral effects and it is clear that the purpose of differentiation of CD8$^+$ T cells is to create an antiviral effector cell, the finding of cells at the end of the primary response that can rapidly produce IL-2 has been 'considered to reflect maximum functional differentiation' (25). Thus, one of the difficulties in assigning relative positions to T_{CM} and T_{EM} in the developmental pathway of antigen-stimulated CD8$^+$ T cells is that there is no widely accepted stepwise pattern of acquisition of cytokine-producing potential during development of CD8$^+$ T cells as there is for CD4$^+$ T cells. When this difficulty is combined with the additional problem of CD62L^{high} CD8$^+$ T_{CM} being capable of producing only IL-2 in some studies (26) and both IL-2 and IFN-γ in other studies (20), one is left with few phenotypic markers that would allow characterization of the relative differentiation of the CD8$^+$ T_{CM} and T_{EM} subsets. Perhaps the definition of the T_{CM} subset based on homing receptor expression is not sufficiently stringent, or differentiation within the CD8$^+$ T-cell lineage is not as defined or synchronously regulated as in the CD4$^+$ T-cell lineage.

The adoptive transfer experiments, however, have provided one means by which the T_{CM} subset can be understood as being less differentiated than the T_{EM} subset, with the ability of T_{CM} but not T_{EM} to mediate a secondary proliferative response. Replication is the essential property of a naïve lymphocyte in a clonal immune system. The development of a subset of antigen-activated CD8$^+$ T cells that retains this capability must occur before development of subsets that have effector activity because the former can produce the latter but the latter cannot generate the former. Therefore, to understand how the T_{CM} develops, one must know the signals that cause the effector differentiation of CD8$^+$ T cells. These are the signals that T_{CM} must modify or avoid during their development and replication. When one accepts that the T_{CM} subset is less differentiated than the T_{EM} subset, it follows that the simplest model for T_{CM} development is a pathway that allows for clonal expansion without effector differentiation.

Evidence for the role of transcriptional repressors of IL-2 signaling in the development of memory CD8$^+$ T cells

IL-2 and IL-15, which stimulate T cells through receptors that share β and common γ chain (γ_c) subunits, cause CD8$^+$ T cells to proliferate and differentiate to T_{EFF} with cytolytic activity and a capacity to produce effector cytokines. Whether

IL-15 always causes differentiation or not is not clear, but it has been shown to do so when CD8$^+$ T cells were re-stimulated in the presence of this cytokine (12). Therefore, for CD8$^+$ T cells to regulate their differentiation, they would need at least to control signals induced by IL-2 and possibly IL-15. A potential means for cell autonomous regulation of IL-2 responses was initially suggested by studies (27) of a transcriptional repressor, BCL-6, expressed by the germinal center B cell. Ectopic expression of BCL-6 in the murine B lymphoma BCL-1 prevented IL-2 from causing the differentiation of these cells to immunoglobulin M (IgM)-secreting plasma cells. This effect of BCL-6 was mediated by repression of signal transducer and activator of transcription 3 (Stat3)-dependent expression of B-lymphocyte-induced maturation protein-1 (Blimp-1), the master regulator of plasma cell differentiation. These and similar findings by Staudt and his colleagues (28) indicated that by arresting terminal differentiation, BCL-6 enables the germinal center B cell to undergo iterative cycles of expansion, somatic hypermutation of Ig genes, and selection for high affinity variants. At a more general level, they also demonstrated that the immune system has a mechanism for controlling cytokine-driven effector differentiation. Although this finding related to B cells, the frequent parallels between the B and T-cell pathways of activation and differentiation at least made plausible the suggestion that CD8$^+$ T cells might also control IL-2-induced differentiation by a similar mechanism. Thus, it was suggested that in the developmental pathway triggered by antigenic stimulation of naïve CD8$^+$ T cells, there is a stage of self-renewal in which cells were restrained from differentiating into effector cells by a BCL-6-like transcriptional repressor (1). This pool of self-renewing cells could then serve as an inexhaustible source of T_{EFF} during persistent infections and as the precursor for the T_{CM} subset that mediates memory responses for intermittent infections.

This hypothesis for active repression of effector differentiation in the development of memory CD8$^+$ T cells has been tested by two groups, one examining a potential role for BCL-6 itself (29, 30) and the other evaluating the participation of a paralogue, BCL6b (originally termed BAZF) (31). BCL-6 has been shown to be expressed as protein in germinal center B cells and, curiously, in cortical thymocytes (32), although no abnormality in thymocyte development has been reported in BCL-6-deficient mice (33–35). Mice with targeted interruption of the *BCL6* gene do not develop germinal centers, indicating an essential role in the germinal center B-cell. They also develop a spontaneous inflammatory disease that is characterized by infiltration of the heart, spleen, gut, liver, and skin with monocytes, eosinophils, and CD4$^+$ Th2 cells. The cause of this process is thought to be dysregulated secretion of chemokines by macrophages (36). Even though an underlying abnormality of lymphocytes is not thought to be basis for the inflammation, it presents a problem when interpreting the responses of memory CD8$^+$ T cells in BCL-6$^{-/-}$ mice.

Both loss-of-function BCL-6$^{-/-}$ mice and gain-of-function BCL-6 transgenic mice have been evaluated (29, 30). In studies using vaccinia/ovalbumin (OVA), there was either no significant difference or only a 50% decrease in the percentage of CD8$^+$ T cells that were OVA-specific 10 weeks post-infection in BCL-6$^{-/-}$ mice relative to the response of normal controls. The function of the BCL-6$^{-/-}$ memory cells was apparently unimpaired in that the fold increase in epitope-specific CD8$^+$ T cells after boosting with OVA peptide did not differ between wildtype and BCL-6$^{-/-}$ mice. In contrast, in mice with the BCL-6 transgene, which was regulated by the *lck* proximal promoter that is usually employed to limit expression to thymocytes, there was enhanced homeostatic expansion of CD8$^+$ T cells in lymphopenic mice and a twofold increase in OVA-specific CD8$^+$ T cells 10 weeks after infection with vaccinia/OVA. The secondary response of the BCL-6 transgenic mice was also higher than in the wildtype mice. These results indicate that the forced expression of BCL-6 in CD8$^+$ T cells promotes their primary and secondary responses, suggesting that suppressing IL-2-induced differentiation may indeed have a role. However, the loss-of-function studies with BCL-6$^{-/-}$ mice did not show a marked reduction in the expansion of CD8$^+$ T cells, and these findings are ambiguous because of the immunological abnormality contributing to spontaneous inflammatory disease in these mice. Thus, a transcriptional repressor of IL-2-induced differentiation may promote the responses of memory CD8$^+$ T cells, but the evidence that BCL-6 is the relevant transcription factor is not conclusive.

The second approach assessed the possible role of the BCL-6 paralogue BCL6b (31). The original report identifying the *BCL6b* gene by its cross-hybridization with BCL-6 cDNA showed that it was expressed in several non-immune organs, such as the heart and lungs, and in splenocytes activated with phorbol ester and ionomycin (37). The participation of BCL6b in the function of memory CD8$^+$ T cells was first suggested by the finding that human and murine T_{CM} and T_{EM} had 10- to 20-fold more BCL6b mRNA than did naïve CD8$^+$ T cells. This increase reflected expression of BCL6b in a small subpopulation of memory cells, but it has not been possible to isolate these cells for further characterization. Mice in which the *BCL6b* gene had been interrupted had normal numbers of CD4$^+$ and CD8$^+$ T cells in the spleen

and lymph nodes, although there was a small decrease in the number of CD4 and CD8 single positive thymocytes. Despite expression of BCL6b in the heart and lungs of wildtype mice, the BCL6b$^{-/-}$ mice exhibited no cardiac or pulmonary abnormalities and did not exhibit spontaneous autoimmune or inflammatory problems. The humoral response of BCL6b$^{-/-}$ mice to a T-dependent antigen was normal with respect to specific IgG1 and IgG2a antibody titers and the generation of germinal center B cells. Therefore, there was normal development in the BCL6b-deficient mice of CD4$^+$ T cells that provide help to B cells, which is dependent on the expression of CD40L, a means by which CD4$^+$ T cells also facilitate development of memory CD8$^+$ T cells.

Primary CD8$^+$ T-cell responses to infection with vaccinia or influenza in BCL6b$^{-/-}$ mice were normal, as was the maintenance of memory CD8$^+$ T cells up to 10 weeks post-infection. However, the secondary response to intranasal influenza infection was diminished in the BCL6b$^{-/-}$ mice. Following a normal initial increase in influenza-specific CD8$^+$ T cells in the BCL6b$^{-/-}$ mice, the response plateaued at a level in the mediastinal lymph nodes which was only one-third that of the peak response in BCL6b$^{+/+}$ mice. There was a corresponding decrease in the number of influenza-specific CD8$^+$ T cells accumulating in the lungs of the BCL6b-deficient mice, as would be expected since replicating cells in the lymph nodes are the precursors of the T_{EFF} in infected peripheral tissue. BCL6b-deficient memory cells that were adoptively transferred into wildtype mice also exhibited an impaired response to influenza infection. Therefore, BCL6b is required for the heightened magnitude rather than the accelerated kinetics of the secondary response. This finding suggests that it is not required for the development or maintenance of memory CD8$^+$ T cells that mediate replication but is required for their continued secondary expansion in the lymphoid tissue. BCL6b may enhance expansion of memory cells by suppressing IL-2 signaling to inhibit effector differentiation, which would allow cells to continue to replicate and accumulate in the draining lymph node. This function would be analogous to that of BCL-6 in the germinal center B cell.

The clear demonstrations of a defect in the memory response of CD8$^+$ T cells in BCL6b$^{-/-}$ mice and of enhancement of the response in BCL-6 transgenic mice indicate that a transcriptional repressor of IL-2 signaling has a positive role. The distinct abnormalities in both *in vitro* and *in vivo* secondary responses in the BCL6b$^{-/-}$ mice and the less clear-cut findings in the BCL-6$^{-/-}$ mice suggest that BCL6b is the relevant transcriptional repressor. Thus, as was initially proposed, a BCL-6-like transcriptional repressor does contribute to the memory response of CD8$^+$ T cells. The contribution is less than originally envisioned, since only the full potential of memory cells for proliferation was affected by BCL6b, not their development, so that active repression of IL-2-induced differentiation by a BCL-6-like transcription factor was not absolutely necessary for the generation of the self-renewing population of antigen-stimulated CD8$^+$ T cells. However, an *in vitro* analysis of whether BCL6b could separate the growth and differentiation effects of IL-2 on CD8$^+$ T cells provided another clue that supported the stem cell model of development, which was that IL-2 may not have a non-redundant role in the clonal expansion of antigen-stimulated CD8$^+$ T cells.

The thought that BCL6b could be selective in suppressing only the differentiation-inducing effects of IL-2 was prompted by a consideration of how it recognizes its target genes. DNA recognition by BCL-6 and BCL6b is mediated by six and five, respectively, Krüppel-type, C-terminal zinc fingers that are 94% identical between the two proteins. The consensus DNA element recognized by these transcriptional repressors is similar to the core TTC(T/C)N(G/A)GAA IFN-γ-activated sequence motif to which the Stat family of transcription factors binds. Consistent with this observation, BCL-6 repressed transcription from reporter constructs containing Stat3 (27, 38) or Stat6 (33, 39) elements. Since it is likely that the zinc finger domains of BCL-6 and BCL6b interact with Stat elements in their target genes in a manner distinct from that of the Stat proteins, it seemed possible that these transcriptional repressors could selectively inhibit the IL-2- and Stat-activated genes involved in differentiation but not the genes involved in cellular replication. This uncoupling of these two processes would permit self-renewal to occur in IL-2-stimulated T cells, as is thought to occur in the germinal center B cell. This possibility was also consistent with the selective inhibition of B-cell differentiation and not of proliferation, when BCL-6 was ectopically expressed in primary murine B cells stimulated with CD40 and various cytokines acting through receptors containing the γ_c chain (27). However, when BCL6b was assessed for its effects on IL-2 responses by primary murine CD8$^+$ T cells *in vitro*, it was found to suppress proliferation induced by the cytokine (31). Therefore, BCL6b is not capable of distinguishing between IL-2 target genes involved in proliferation and differentiation. The original hypothesis of the relatively undifferentiated state of the T_{CM} subset being dependent on selective repression of some effects of IL-2 signaling was not correct. However, these results did present an apparent paradox in that BCL6b suppressed IL-2-induced proliferation *in vitro*, but promoted the expansion of memory CD8$^+$ T cells *in vivo*. The

simplest explanation for resolving this paradox would be that antigen-induced proliferation of $CD8^+$ T cells is largely IL-2-independent.

A response of $CD8^+$ T cells in the absence of IL-2/IL-15 signaling?

The question of whether $CD8^+$ T cells can have significant clonal expansion without signaling through the IL-2R has not been clearly resolved, but the preponderance of evidence suggests that this may occur and that $CD8^+$ T-cell growth in the absence of signaling through the IL-2/IL-15 receptor actually may account for most of their expansion in a primary and perhaps a secondary response (*Table* 2). The first analysis of the primary antiviral $CD8^+$ T-cell response in the absence of IL-2 was more than 10 years ago, and it concluded that infection with vaccinia or LCMV of mice in which the *IL-2* gene had been functionally inactivated led to the development of normal numbers of virus-specific cytotoxic T lymphocytes (CTLs), as measured by *ex vivo* lysis of peptide-loaded target cells (40). The possibility of redundant cytokine functions was considered, and the study was repeated with mice that were both IL-2- and IL-4-deficient. CTLs were still generated in response to an infection with LCMV but not vaccinia in double-deficient mice (41). The authors recognized that a potential role of IL-15 had not been excluded. Another group at approximately this time also evaluated the response of $IL\text{-}2^{-/-}$ mice to infection with LCMV, and they found diminished production of CTLs and of IFN-γ (42). Although no reasons were offered to account for the disparate outcomes of these two sets of experiments, both groups of investigators acknowledged the potentially confounding effect of lymphoproliferative disease in the IL-2-deficient mice, which we now know to be caused by the absence of the Foxp3-expressing regulatory T-cell lineage. These early studies were also hindered by being dependent on antigen-specific effector functions of $CD8^+$ T cells to detect their presence, which could mean that T_{CM} were missed. Both problems were avoided in a study of the effects of IL-2 deficiency in $CD8^+$ T cells expressing a transgenic TCR specific for the class I-restricted influenza nucleoprotein peptide (43). Although the main aim of the study was to evaluate thymic development in the absence of IL-2, which was normal, it also examined the response of the TCR-transgenic $IL\text{-}2^{-/-}$ $CD8^+$ T cells to peptide immunization and found normal expansion but absence of CTL effector activity; the development of CTL effector function required IL-2. This is the first report suggesting that effector differentiation, not replication, is the essential, non-redundant role for IL-2 in the developmental pathway of antigen-stimulated $CD8^+$ T cells. However, the *in vivo* peptide stimulation of the $CD8^+$ T cells was not physiological, lacking the inflammatory components of a normal viral infection, and the role of IL-2 and/or IL-15 in the primary response of $CD8^+$ T cells to viral infection remained unclear.

In 2002, this question was examined again by measuring the response of CD25-deficient or IL-2-deficient adoptively transferred OT-I $CD8^+$ T cells specific for the SIINFEKL peptide (44). Infection of recipient mice with vesicular stomatitis virus expressing this peptide induced equivalent expansion of wild type, CD25-deficient, and IL-2-deficient OT-I cells in secondary lymphoid organs. Interestingly, expansion of the $CD8^+$ T cells, especially in the *lamina propria* but also in other non-lymphoid organs, was diminished in the absence of IL-2

***Table* 2. Studies of the role of IL-2/IL-15 signaling in the primary and secondary responses of $CD8^+$ T cells**

Study	Genetic deficiency	Immunological challenge	Clonal expansion*	Effector differentiation	Secondary expansion *in vivo*
Kundig *et al.* (40)	IL-2	LCMV, vaccinia		Normal	Not assessed
Kramer *et al.* (43)	IL-2	Peptide	Yes	Absent	Not assessed
Bachmann *et al.* (41)	IL-2	LCMV		Normal	Not assessed
	IL-4	"		Normal	Not assessed
	IL-2/4	"		Slight reduction	Not assessed
	IL-2	Vaccinia		Reduced	Not assessed
	IL-4	"		Normal	Not assessed
	IL-2/4	"		Absent	Not assessed
Cousens *et al.* (42)	IL-2	LCMV		Absent	Not assessed
D'Souza *et al.* (44)	IL-2	VSV	Lymphoid-yes Non-lymphoid-no	Not assessed	Not assessed
	CD25	"	"	Not assessed	Not assessed
Yu *et al.* (48)	CD122	Vaccinia/Sindbis	Yes	Absent	Yes

*In the early studies, class I tetramers were not available. In experiments not involving TCR transgenic mice, clonal expansion could not be determined independently of effector function.

signaling, suggesting that IL-2 may promote growth mainly in CD8$^+$ T cells that have left secondary lymphoid tissues, possibly linking IL-2 stimulation to a commitment to effector differentiation. The converse, that a response to IL-2 is not required for expansion of the relatively less differentiated CD8$^+$ T cells in secondary lymphoid tissues, also holds. A subsequent study using similar methods concluded that IL-2 is not required for the early primary proliferative response of CD8$^+$ T cells (45). If, as seems likely, there is a temporal progression from an initial phase of expansion of antigen-specific naïve CD8$^+$ T cells in the secondary lymphoid tissues to a later phase of expansion of responding cells in the periphery, this second study reinforces the possibility that IL-2 promotes only the growth of CD8$^+$ T cells that have committed to effector differentiation. Thus, relatively undifferentiated CD8$^+$ T cells, perhaps the pre-T_{CM}, may proliferate in an IL-2-independent manner, and more differentiated cells, T_{EM} and T_{EFF}, may require IL-2 for proliferation. Other inferences regarding the role of IL-2 in the development of T_{CM}, T_{EM}, and T_{EFF} cannot be made, because the study did not assess the effector functions of CD8$^+$ T cells that expanded with or without IL-2 signaling, their expression of homing receptors or ligands, or their ability to mediate secondary replicative responses. The report also could not exclude the participation of IL-15, and some studies (12, 46) but not others (47) have presented findings suggesting that IL-15 promotes the primary response of naïve CD8$^+$ T cells. However, these studies with CD25- and IL-2-deficient CD8$^+$ T cells do overcome three problems in early analyses of the role of IL-2 in the response of CD8$^+$ T cells: by the use of TCR transgenic T cells and their adoptive transfer into normal recipients, the spontaneous lymphoproliferative disease associated with absence of IL-2 signaling was no longer a confounding factor; by enumerating responding CD8$^+$ T cells by their physical presence instead of functional response, the measurement of the response did not require that the responding cells have acquired effector function; and the use of viral vectors to express peptide epitopes presented the CD8$^+$ T cells with a physiological stimulus. One could conclude unambiguously from the results of these studies that IL-2 signaling does not had a non-redundant function in the clonal expansion of naïve CD8$^+$ T cells within the secondary lymphoid tissues.

The three questions of whether IL-15 signaling can compensate for the absence of IL-2 in the expansion of naïve CD8$^+$ T cells, whether the differentiation of responding CD8$^+$ T cells to effector cells is dependent on IL-2 or IL-15 or not, and whether the development of memory cells requires stimulation by these cytokines or not were addressed by Malek and his colleagues in 2003 (48). To exclude effects of IL-2 and IL-15, mice were developed in which CD122, the shared β-subunit of the IL-2/IL-15 receptor, was deleted by targeted interruption of the *CD122* gene. To compensate for the requirement for IL-2 signaling in the development of Foxp3-expressing regulatory T cells, the mice also had a transgene expressing CD122 under control of the *lck* proximal promoter, which restricted expression of CD122 to developing thymocytes. Consistent with the success of this strategy, the mice did not have lymphoproliferative disease, and their peripheral mature T cells did not respond to IL-2 *in vitro*, demonstrating that the CD122 transgene was expressed only in the thymocytes. Remarkably, these mice demonstrated normal expansion of antigen-specific CD8$^+$ T cells to primary infection with a recombinant vaccinia virus expressing the immunodominant S510 epitope from the spike protein of mouse hepatitis virus. Further, these antigen-specific cells differed from those that developed in wild type mice in that they lacked two effector functions, the ability to produce IFN-γ when stimulated with antigen *in vitro* and, in some assays, the ability to kill antigen-expressing target cells immediately *ex vivo*. Therefore, in the absence of IL-2 and IL-15 signaling, CD8$^+$ T cells clonally expanded and replication was not accompanied by the acquisition of effector function. This study made another critical observation. The memory CD8$^+$ T cells that developed in the CD122-deficient mice expanded normally when challenged with a secondary infection with Sindbis virus expressing the same S510 epitope. Since effector CD8$^+$ T cells were not generated in the primary response, the precursors of the memory cells that mediated secondary replicative responses, presumably the T_{CM} subset, must have developed directly from naïve CD8$^+$ T cells. Although the complex genetic strategy used to generate these mice may lead to concerns about a possible 'leakiness' of the transgene expressing CD122, the absence of effector function in the virus-specific CD122-deficient CD8$^+$ T cells suggests that signaling *via* the IL-2/IL-15 receptor had not occurred and that expression of the CD122 transgene was appropriately restricted to developing thymocytes.

Implications of IL-2/IL-15-independent signaling for models of memory CD8$^+$ T-cell development

These studies on the role of IL-2 and IL-15 in the response of CD8$^+$ T cells to viral infections lead to four conclusions that place constraints on the pathways that one can propose to account for the development of T_{CM}, T_{EM}, and T_{EFF}. First, clonal expansion that produces the expanded pool of memory

cells that is responsible for the secondary proliferative response is not mediated by IL-2 or IL-15. This consideration is based on the finding that memory CD8$^+$ T cells in CD122-deficient mice mediated a normal secondary replicative response (48). Thus, an assumption derived from over 20 years of *in vitro* studies that IL-2 is the essential T-cell growth factor must be revised (49), and the mechanism by which CD8$^+$ T cells expand in secondary lymphoid organs needs to be defined, as this is the cellular response that establishes replicative memory.

Second, clonal expansion and effector differentiation are not coupled when expansion is driven by this unknown mechanism that is independent of IL-2 and IL-15. This finding suggests that the unknown means for CD8$^+$ T-cell growth permits self-renewal, at least in the sense that the progeny of cells dividing in response to this unknown signal are not obligated to become effector cells and maintain the replicative potential and undifferentiated phenotype of the parental cell. Presumably, this is the mechanism for clonal expansion that avoids exhaustion of antigen-selected clones.

Third, expansion that is mediated by IL-2 is coupled to effector differentiation. By analogy to myelomonocytic differentiation, IL-2 may be to the CD8$^+$ T cell as colony-stimulating factors are to developing granulocytes and monocytes. Each cell cycle of myelomonocytic cells growing in response to colony stimulating factors, or of CD8$^+$ T-cell responding to IL-2, is associated with further differentiation to an effector cell. The study by Malek and his colleagues (48) further suggests that IL-2 and/or IL-15 is absolutely required for at least some aspects of effector differentiation, although under unusual circumstances and with *in vitro* studies, other cytokines signaling through other receptors using the γ_c chain, such as IL-4, can drive effector differentiation of the CD8$^+$ T-cell. Whether this occurs with most viral infections *in vivo*, which tend to induce a Th1 response, may not be likely.

The cells that replicate and differentiate in response to IL-2 and/or IL-15 therefore must be the precursors of T_{EM}, since the memory cells generated from CD122-deficient CD8$^+$ T cells lacked the effector functions characteristic of this subset. Unfortunately, analysis of the expression of homing receptors, which could have supported this conclusion, was not conducted. The distinction between T_{EM} and T_{EFF} is, in essence, operational with the former being defined as cells with effector function that persist into the memory phase. The molecular distinction between T_{EM} and T_{EFF} may be the expression by the former of the IL-7Rα, since IL-7 is required for the CD8$^+$ T cells responding in the primary response to persist into the memory phase (14, 50). Thus, IL-2/IL-15 may broadly regulate the development of cells with effector function, while other, perhaps 'inflammatory', signals that regulate the expression of the IL-7Rα may determine whether an IL-2-stimulated CD8$^+$ T cell becomes a T_{EM} or a T_{EFF}.

Fourth, if clonal expansion of CD8$^+$ T cells *via* the IL-2/IL-15-independent pathway is associated with absence of effector differentiation and is responsible for the T_{CM} subset and if effector differentiation is mediated by the two transcription factors T-bet (51) and eomesodermin (52), then the T_{CM} derived from the IL-2/IL-15-independent subset may not have expressed T-bet or eomesodermin. If this assumption is correct, then the memory cells that mediate a replicative secondary response may not be dependent on IL-15 for their maintenance. This possibility is based on the recent finding that T-bet and eomesodermin are required for the upregulation of CD122 during activation of CD8$^+$ T cells (53). Increased expression of CD122 had been established as being associated with responsiveness to IL-15. Although IL-15 has been shown to be necessary for the slow replication of memory CD8$^+$ T cells that mediates their long-term persistence (47, 54, 55), two studies suggest that not all memory CD8$^+$ T cells require IL-15 for their maintenance. First, the subset of gp33-specific memory CD8$^+$ T cells that expands in response to a secondary infection with LCMV was maintained after three months in the absence of IL-15, despite a reduction of 90% in total gp33-specific memory CD8$^+$ T cells, relative to the number in IL-15-sufficient mice (47). Second, among the CD44$^+$ memory CD8$^+$ T cells that arise apparently in response to environmental antigens, a subset of CD122^{low} cells were maintained in the absence of IL-15, even while the CD122^{high} subset was markedly depleted (54). This CD44^{high}CD122^{low} IL-15-independent population is not as evident in an elicited memory CD8$^+$ T-cell pool, and it would be interesting to determine whether the residual gp33-specific memory population in the IL-15-deficient mice that mediated a normal secondary response was CD122^{low}. It may be that the cytokine requirements for maintenance may differ among the T_{CM} and T_{EM} subsets of memory cells, with perhaps only the T_{EM} subset being IL-15-dependent, because they have induced T-bet and eomesodermin and upregulated CD122.

It is informative to examine how these four inferences square with the different models for the development of antigen-stimulated CD8$^+$ T cells. First, the finding that T_{CM} can develop in the absence of T_{EM} indicates that the linear differentiation model, which places T_{EFF} and T_{EM} before T_{CM} in the developmental pathway, must be modified to include a direct naïve T-cell to T_{CM} developmental step. This step is consistent with the progressive differentiation model, the

observations of Bachmann *et al.* (20), Bouneaud *et al.* (21) and Marzo *et al.* (23), and the stem cell model for antigen-stimulated CD8$^+$ T-cell development (1). Second, the development of T_{CM} but not T_{EM} in the absence of IL-2/IL-15 signaling is also consistent, in part, with the prediction of the progressive differentiation model that T_{EM} and T_{EFF} development require 'stronger' signals. However, it may be that 'different signals' would more closely reflect the respective requirements of these subsets for development. Third, as was inherent in the original description of the T_{CM} and T_{EM} subsets in which the latter but not the former had potential effector functions, and as IL-2 induces effector differentiation of CD8$^+$ T cells, the T_{CM} subset can and perhaps must develop independently of the differentiating effects of IL-2/IL-15. The mechanism for this clonal expansion probably is the basis for the essential stem cell characteristic of antigen-stimulated CD8$^+$ T cells of maintaining the production of differentiated cells in persistent viral infections.

An essential next step: definition of the IL-2/IL-15-independent mechanism for clonal expansion of CD8$^+$ T cells

A critical problem now is to discover the means by which the CD8$^+$ T cell grows in the absence of signaling through the IL-2/IL-15 receptor. The phenotypic requirements for such growth are threefold: it must be dependent on ligation of the TCR and, in some way, on the activation of dendritic cells by microbial products acting on Toll-like receptors and possibly by ligation of CD40; it must not induce the production of IL-2, so as not to induce differentiation to T_{EFF} or T_{EM}; and it must maintain the expression of the receptors and ligands required for residence in secondary lymphoid tissue and of the IL-7Rα for survival. There is not an absolute requirement for a BCL-6/BCL6b-like transcriptional repressor of IL-2-induced differentiation to create this self-renewing stem cell, because this as yet undefined IL-2/IL-15-independent pathway of CD8$^+$ T-cell clonal expansion obviates the need to uncouple cytokine-induced proliferation and differentiation. However, it is possible that when the number of antigen-specific CD4$^+$ and CD8$^+$ T cells reaches a critical density within the T-cell zone of the responding secondary lymphoid organ, paracrine IL-2 could begin to drive effector differentiation and restrict the expansion of the self-renewing pool of CD8$^+$ T cells. In this circumstance, the expression of BCL6b by self-renewing CD8$^+$ T cells would insulate them from IL-2 and allow their continued IL-2-independent growth and retention in the lymphoid environment (*Fig. 1*). This process would increase the pool of self-renewing cells and, since these are the precursors of T_{EFF}, correspondingly increase the rate at which effector cells are generated.

In summary, the following pathway is proposed to account for the observations that have been referred to in this review. Naïve CD8$^+$ T cells are 'programmed' by interaction with appropriately activated mature dendritic cells that present the relevant antigen. The programming allows the CD8$^+$ T cells either to respond to an unidentified ligand that is present on dendritic cells, which induces proliferation without differentiation, or to IL-2/IL-15, which induces proliferation that is coupled to differentiation. The former constitute a self-renewing population from which cells can 'spin-off' and respond to IL-2, either autocrine or paracrine. These IL-2-responding cells then proliferate in a manner that is coupled to differentiation to T_{EFF} and leave the lymphoid environment. At the end of the primary response, the self-renewing population gives rise to resting T_{CM}, and the IL-2-responding subset yields T_{EM}. Both require IL-7 for survival, but only the T_{EM} require IL-15 to maintain their numbers. In a secondary response, the high precursor frequency of both CD4$^+$ and CD8$^+$ T_{CM} causes a rapid build-up of replicating cells that would produce relatively large amounts of IL-2, which, by driving differentiation, could limit the expansion of the self-renewing pool. BCL6b represses the effects of this IL-2, blocking differentiation, allowing further expansion of the self-renewing pool and enabling generation of a larger number of T_{EFF} than occurs in the primary response.

Clearly, the critical element needed to support this proposed pathway is the mechanism of IL-2/IL-15-independent clonal expansion. From this basic finding, the various other predictions can then be tested.

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