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## Diversity in T cell memory: An embarrassment of riches

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### Abstract

The adaptive immune response meets the needs of the organism to generate effector cells capable of controlling pathogens, but also leads to production of memory cells, which mediate more effective protection during rechallenge. In this review we focus on the generation, maintenance and function of memory T cells, with a special emphasis on the increasing evidence for great diversity among functional memory T cell subsets.

### Introduction: Do we have a definition of effector and memory T cells?

During a typical immune response to an acute pathogen, antigen specific cells are activated, proliferate vigorously and expand extensively. This expansion phase yields a large population of effector T cells, most of which will die in the subsequent contraction phase of the response (Fig. 1). However, the expansion phase also yields cells that will eventually form the memory cell pool - primed cells maintained long term after immunization. Immune memory indicates a qualitatively and/or quantitatively distinct immune response upon successive (but interrupted) exposures to antigen. This leads to an improved secondary immune response compared to the primary response – “improved” in this context usually means greater in magnitude, faster, more sensitive to low doses of antigen and more effective in the diversity or complexity of secondary effectors (Kaech and Wherry, 2007) (Harty and Badovinac, 2008; Williams and Bevan, 2007).

The memory cell pool is not monolithic but rather contains diverse and pliable populations. Two broad categories of memory cells are effector-memory (Tem) and central-memory (Tcm) (being CD62L<sup>lo</sup>CCR7<sup>lo</sup> and CD62L<sup>hi</sup>CCR7<sup>hi</sup>, respectively) (Sallusto et al., 1999). This characterization, together with earlier studies, suggest that phenotypically defined subsets possessed distinct functional properties (Sallusto et al., 1999) (Hamann et al., 1997), but subsequent work has only added to the range of phenotypic and functional memory subsets which can be identified. So, are there features we can use to clearly define memory cells (and their subsets), and distinguish these from effector cells? A minimal definition of memory cells would be the population that persists long term after antigen clearance (whereas “typical” effector cells do not). However, there is no consensus on the minimum longevity sufficient to classify as “memory”, and this definition includes no functional properties. A commonly used additional criteria is the ability of “typical” memory (but not effector) cells to undergo recall proliferation and differentiation into secondary effector and memory populations. Use of these two parameters (longevity and proliferative potential) allows demarcation of idealized naïve, effector, Tem and Tcm cell populations (Fig 2a). Tem cells are generally considered to have a more limited lifespan and weaker proliferative potential, compared to their Tcm cell counterparts. However, analysis of actual post-activation populations illustrates much greater

diversity in survival and recall potentials (Fig 2b) as well as diverse subsets defined by other phenotypic and functional markers in lymphoid and non-lymphoid sites (see, e.g. (Hikono et al., 2007; Masopust et al., 2006b)). Some cells represented in Fig 2b arise in certain tissue sites, or distinct stages of the immune response but this chart helps illustrate that memory subsets, as well as the properties of “effector” and “memory” cells, are not sharply distinct. Nor will one pair of criteria accurately define the populations potentially generated in an immune response: a different chart (with just as much diversity) could be produced plotting other features – such as cytolytic activity versus longevity for CD8<sup>+</sup> T cell populations. Without appreciating the diversity of the post-activation T cell pool, we may miss critical elements in the armaments induced during adaptive immunity. Our goal here is to discuss how different populations of post-activation T cells are generated, and how this diverse pool may all contribute to distinct aspects of recall immune responses and (a distinct feature) protective immunity. This review will focus exclusively on T cells and, because of space constraints will focus mainly on findings from acute antigen exposure models. The effects of chronic and persistent infections on T cell memory have been discussed in recent reviews (Harty and Badovinac, 2008; Kaech and Wherry, 2007; Williams and Bevan, 2007).

### ***The choice between “effector” and “memory” differentiation: One cell to yield them all?***

Activation of T cells generates a pool with potential to enter the effector or the memory cell population. A key question, intensely investigated in recent years, is what influences the effector versus memory decision. The simplest model of all would be that effector cells and memory cells differentiate from distinct subsets of naïve T cells. However, adoptively transferred single naïve CD8<sup>+</sup> T cells can give rise to both effector and diverse memory populations (Stemberger et al., 2007), a result reinforced by an alternative innovative method of “cellular barcoding” (Schepers et al., 2008) (Schumacher personal communication). Such studies, by necessity, utilized T cell receptor (TCR) transgenic T cells, so it is unclear whether these results will always be generalizable. Indeed, recent reports argue that sub-optimal TCR stimulation can lead to T cell elimination after the effector phase (Teixeiro et al., 2009; Williams et al., 2008), suggesting that there may be instances where individual clones contribute to the effector but not memory pools (and, potentially, the reverse).

An extension of the “one cell-multiple fates” model has been proposed by Reiner’s group based on their studies of asymmetric cell division during the initial T cell response (Chang et al., 2007). This report found differential segregation of receptors and signaling molecules between the first two daughter cells of activated naïve T cells, and suggested they may differ in their capacity to produce effector versus memory pools (Fig 3). At its most extreme, this model would indicate the fate of the effector versus memory “lineages” is sealed early in the response, driven by differential gene expression programs. In contrast to this strict model, recent work argues gene expression of “effector-like” molecules (such as Granzyme B in CD8<sup>+</sup> T cells and IFN- $\gamma$  in CD4<sup>+</sup> T cells) in cells that later join the memory pool (as well as in effector cells) (Bannard et al., 2009; Harrington et al., 2008; Lohning et al., 2008; Maris et al., 2003). These and other reports fit best with a form of linear differentiation model in which activated T cells all pass through an “early effector” phase during which genes for several effector molecules are expressed. However, this does not exclude the possibility that cells produced by the first few divisions have different capacities to join the effector versus memory pool later on (Fig 3). In addition, however, “environmental” cues such as inflammatory cytokines can dramatically affect subsequent differentiation of the activated T cell population (Fig. 3), as will be discussed next.

## Everything in moderation: better memory by avoiding over-exertion?

A key question is when competent memory T cell precursors emerge after activation of naïve T cells. Studies on CD8<sup>+</sup> T cells well before the peak of expansion after primary acute LCMV infection suggest low expression of the molecule KLRG-1 identifies cells containing the memory precursor pool (Sarkar et al., 2008). Although the reliability of KLRG-1 expression as a memory precursor marker in other infection models is unclear, this approach has been useful to study memory cell generation in the LCMV system. As early as 4.5 days after primary LCMV infection, some KLRG-1<sup>lo</sup> cells have already reacquired the ability to make interleukin-2 (IL-2) and go on to show enhanced survival and recall proliferation potential (Sarkar et al., 2008). These cells also show enhanced ability to subsequently upregulate IL-7R $\alpha$ , which is important for memory T cell survival (Buentke et al., 2006; Kaech et al., 2003; Schluns et al., 2000) and to differentiate into a long-lived pool of T<sub>cm</sub> cells. So what conditions favor generation and/or survival of memory precursors? Current models suggest a delicate balance between sufficient but not over-exuberant responses to various stimuli is the key to good memory.

An appealing model for effector or memory choice builds around the idea that sustained stimulatory signals drive cells into a short-lived effector pool, whereas more transient (but sufficient) signals favor generation of memory cells (Intlekofer et al., 2006; Kaech et al., 2002; Lanzavecchia and Sallusto, 2002). The signals discussed here include TCR encounter with peptide-MHC but also the integration of stimuli through various adhesion, costimulatory, and cytokine receptors.

Studies in which the intensity of inflammatory cues is controlled suggest restrained exposure to inflammatory cytokines enhances generation of memory-like cells (Haring et al., 2006; Kaech and Wherry, 2007). Inflammatory cytokines such as type I interferon (IFN-I) and IL-12 operate as “signal 3” (with TCR and costimulation) to promote CD8<sup>+</sup> T cell effector differentiation (Mescher et al., 2006). Studies suggest intense and/or sustained exposure to IL-12 preferentially promotes CD8<sup>+</sup> T cell differentiation toward the effector rather than memory fate (Joshi et al., 2007; Pearce and Shen, 2007). Other inflammatory cues probably work indirectly rather than on the T cell itself, and the impact of cytokine deficiencies can be complex. An instructive example is IFN- $\gamma$ , deficiency of which leads to a substantial block in CD8<sup>+</sup> T cell contraction (Badovinac et al., 2000), yet testing the effects of deficiency of IFN- $\gamma$  receptor selectively on responding CD8<sup>+</sup> T-cells suggests the cytokine offers a competitive advantage for memory T cell generation, in at least some responses (Whitmire et al., 2007).

How changes in the intensity and/or duration of stimuli changes the balance of effector versus memory differentiation is still being discerned. However, evidence points to a key role for differential expression of the transcription factor T-bet, well known for supporting “Type-1” T cell differentiation (Glimcher, 2007). Increasing T-bet expression in activated CD8<sup>+</sup> T cells drives differentiation away from memory-like and toward effector-like cells, and T-bet is efficiently induced by stimuli such as IL-12 which promote effector production (Joshi et al., 2007; Pearce and Shen, 2007). Furthermore, recent reports suggest that the transcription factor Blimp-1 is critical for generating mature effector cells (Kallies et al., 2009; Rutishauser et al., 2009). Stimulated Blimp-1 deficient T cells preferentially differentiated into central memory-like cells, curtailing the production of effector cells, a situation which was associated with more efficient immune control of some pathogens (Rutishauser et al., 2009), but not others (Kallies et al., 2009). In B cell differentiation, Blimp-1 and Bcl-6 show mutually antagonistic roles (Calame, 2006), and this may parallel the situation in T cells because Bcl-6 deficient mice exhibit defects in the memory CD8<sup>+</sup> T cell pool (Ichii et al., 2002).

For CD8<sup>+</sup> T cells, there is evidence that terminating antigen-encounter (“early leavers” in the response) enhances production of memory precursors (Sarkar et al., 2008). Cells which enter the response late (“late comers”) may also have some advantages in differentiation into the memory pool (Catron et al., 2006; D'Souza and Hedrick, 2006; van Faassen et al., 2005).

However, studies show that at least some perception of inflammatory cues is required for memory cell differentiation. CD8<sup>+</sup> T cells completely lacking exposure to signal 3 cytokines or deficient for T-bet showed impaired generation of long lived functional memory cells, and T-bet is required for normal expression of CD122 (the beta-chain of the IL-2 and IL-15 receptors) and reactivity to the homeostatic cytokine IL-15 (Intlekofer et al., 2005; Joshi et al., 2007; Shaulov and Murali-Krishna, 2008; Xiao et al., 2009). Indeed, in vitro studies indicate exposure to IL-12 early during T cell priming leads to effective generation of memory CD8<sup>+</sup> T cells (Xiao et al., 2009). Also, it is important to note that in several studies where inflammatory cues are shown to promote effector T cell generation, this did not occur at the expense of generating memory cells, suggesting that the memory differentiation pathway can progress in the face of varied production of effector cells (Cui et al., 2009; Joshi et al., 2007). Prematurely truncating exposure to peptide-MHC ligands may lead to a failure in memory (and also effector) differentiation (Fig 3). Activation of CD8<sup>+</sup> T cells for only a few hours leads to their becoming “unfit” for successful differentiation (Gett et al., 2003; van Stipdonk et al., 2003; Williams and Bevan, 2004), and shortened encounters with dendritic cells (DCs) (due to blocked ICAM-LFA interactions) produces a similar effect (Scholer et al., 2008). Studies with CD4<sup>+</sup> T cells also show that suboptimal and/or short term stimulation leads to production of functionally compromised effector cells and minimal memory generation (Blair and Lefrancois, 2007; Gett et al., 2003; Williams and Bevan, 2004; Williams et al., 2008). At the other extreme, overstimulation of activated T cells (e.g. during chronic infection) can provoke an “exhausted” state, also manifest as impaired function and maintenance (Kaech and Wherry, 2007).

Together, these studies would argue that there is a Goldilocks, “just right” point in which response to TCR cues, inflammatory cytokines (and potentially other cues) supports memory differentiation without pushing the cells too hard into the short lived effector pool (Fig 3). But what does “pushing too hard” mean in molecular terms? Quantitative differences in expression of key transcription factors (e.g. T-bet and Blimp-1, discussed above) may dictate effector versus memory gene expression patterns. In addition, recent reports suggest control of T cell metabolism is important for differentiation towards the long-lived memory pool. T cells need to switch from the anabolic metabolism characteristic of effector cells to adopt the catabolic metabolism of quiescent memory (and naïve) T cells (Jones and Thompson, 2007). Two recent reports show that memory CD8<sup>+</sup> T cells fail to form if this metabolic switch is prevented (Araki et al., 2009; Pearce et al., 2009). Regulating the molecule mTOR is key in this metabolic change, as illustrated by the capacity of the mTOR inhibitor rapamycin (or knockdown of components in the mTORC1 complex) favoring emergence of memory CD8<sup>+</sup> T cells from the effector pool (Araki et al., 2009; Pearce et al., 2009), and a related pathway controlling fatty acid oxidation, is also implicated in the metabolic switch (Pearce et al., 2009). Once again, however, this regulation is a balancing act, as complete blockade of mTOR prevents initial T cell activation and expansion. Such results beg the question of why some cells in the normal response are successful at making the required metabolic switch to differentiate from the effector pool, whereas most cells are not. Via PI3K, mTOR is activated through activation of the TCR and costimulatory ligands and by various cytokine receptors which, as we have seen, can modulate the effector and memory cell differentiation. Further, in vitro assays show that culture of activated CD8<sup>+</sup> T cells with IL-2 sustains an effector phenotype, while IL-15 promotes T<sub>cm</sub> cell-like differentiation (Manjunath et al., 2001; Weninger et al., 2001), these differences being mediated, at least in part, by the intensity of PI3K and mTOR stimulation (Sinclair et al., 2008). Moderation of stimulatory cues may therefore be important for

promoting the metabolic switch (as well as other effects) favoring development of the memory pool.

Whether the same rules apply to boosted memory populations is much less clear. Markers such as KLRG-1 and effector molecules such as Granzyme B are strongly expressed by cells which seed the secondary (and tertiary) memory pools and these very stable populations very slowly, if at all, acquire a Tcm cell phenotype (Jabbari and Harty, 2006; Masopust et al., 2006a; Vezyts et al., 2009). Because most vaccines are boosted (as, probably, most natural infections are reencountered) these data highlight an important gap in our knowledge of normal memory cell pools.

An unresolved issue is whether factors which alter the balance of effector versus memory cell generation do so by causing altered differentiation of a uniformly pliable precursor pool, or whether these stimuli promote expansion or survival of more committed precursors within the activated population (Fig 3). Resolving such questions, which have been carefully discussed in previous reviews (Harty and Badovinac, 2008; Kaech and Wherry, 2007; Williams and Bevan, 2007), will require further analysis of the differentiation potential of T cells generated early in the immune response.

### The contraction phase: making life and death decisions

During contraction short-lived cells are eliminated from the antigen specific population. What is the basis for survival of some cells and death of others? It has become clear that the death pathway during contraction involves a prominent role for the pro-apoptotic factor Bim. Activity of Bim is normally restrained by Bcl-2 (and related molecules), but downregulation of Bcl-2 during activation leaves effector cells vulnerable. T cells lacking Bim undergo relatively normal expansion, but are more resistant to contraction than their wild-type counterparts (Hildeman et al., 2002; Hughes et al., 2008; Prlic and Bevan, 2008; Weant et al., 2008). The transcriptional regulator Id2 has an important role in preserving cells through the expansion phase, as Id2-deficient effector CD8<sup>+</sup> T cells show increased cell death, accompanied by elevated Bim and reduced Bcl-2 expression (Cannarile et al., 2006)

But what makes short-lived cells susceptible to activating apoptotic pathways, whereas other cells emerge unscathed? An initially attractive candidate would be signals from homeostatic cytokines such as IL-7. Expression of IL-7R $\alpha$  (CD127) is downregulated on activated T cells, but is re-expressed on a population of cells late in the expansion phase, which correlates with the KLRG-1<sup>lo</sup> pool (Joshi et al., 2007; Kaech et al., 2003). IL-7 is important for maintenance of the memory pool, and (as discussed above) cues that favor memory T cell generation drive re-expression of CD127. Furthermore, IL-7 (as well as other  $\gamma$ c cytokines) induces expression of Bcl-2, which restrains Bim activity. However, several studies have shown that CD8<sup>+</sup> T cell contraction occurs even in priming situations in which the majority of cells at the expansion peak express IL-7R $\alpha$  (Lacombe et al., 2005), and also that enforced (transgenic) expression of IL-7R $\alpha$  does not rescue activated cells into the long lived pool (Hand et al., 2007; Haring et al., 2008). Hence CD127 expression and reactivity to IL-7 is likely necessary but not sufficient to drive the effector to memory cell transition. Along similar lines, some priming conditions (e.g. DC vaccines) can very rapidly generate cells which exhibit memory properties (including KLRG-1<sup>lo</sup>CD127<sup>hi</sup> phenotype and recall functional potential) – yet these cells contract to a similar extent as cells primed by conventional pathways (Badovinac et al., 2005).

So, are there priming situations where contraction is averted or minimized? Very short term pathogen infection can lead to priming of a CD8<sup>+</sup> T cell response which is modest in size but very effectively undergoes transition to a functional memory pool (Badovinac et al., 2004). The idea that this outcome is (at least in part) due to moderating inflammatory stimuli is further supported by the similar phenotype in IFN- $\gamma$  deficient animals (Badovinac et al., 2000) (Harty

and Badovinac, 2008). Furthermore, there is generally less contraction after secondary responses (Badovinac et al., 2003; Masopust et al., 2001a) and this effect is even more dramatic after tertiary stimulation (Masopust et al., 2006a; Vezys et al., 2009). Such findings might be related to more rapid elimination of antigen and reduced intensity or duration of inflammatory cytokines such as IFN- $\gamma$  due to the high frequency of antigen reactive cells. Interestingly, the phenotype of the durable memory cells from boosting is similar to effector memory or even effector cells (Jabbari and Harty, 2006; Masopust et al., 2006a; Masopust et al., 2006b), which contrasts with models in which differentiation to Tcm cell phenotype predicts optimal longevity.

What about the opposite situation; priming situations which leads to more extensive contraction? As discussed earlier, insufficient duration or quality of TCR and other stimuli can yield effector cells which contract without leaving a memory pool. For the CD4<sup>+</sup> T cell pool, this is also seen in situations of excessive competition with naïve cells of the same specificity (Blair and Lefrancois, 2007; Hataye et al., 2006; Whitmire et al., 2008). Strangely enough, for CD8<sup>+</sup> T cells abnormally high naïve T cell precursor frequencies and short term antigen exposure seem to accelerate rather than block production of functional memory cells without augmenting contraction (Badovinac et al., 2007). The basis for these interesting differences between CD4<sup>+</sup> and CD8<sup>+</sup> T cells is unclear, but highlights the risks of interpreting studies using antigen specific naïve T-cells at non-physiologically high frequencies.

### The issue of “not-so-short lived” effectors

Despite the substantial decline in antigen specific effector T cells during contraction, some cells with this phenotype are found a considerable time after clearance of antigen (many months in the spleen, and even longer in other tissue sites – see later). Further, adoptive transfer approaches show that effector-like cells actually have a fairly leisurely decay rate. For example, T cells isolated from the expansion phase as “effector” versus “memory precursor” pools (using markers such as IL-7R $\alpha$  or KLRG-1) differ only slightly (~3 fold) in their persistence at 4-6 weeks (Kaech et al., 2003; Sarkar et al., 2008), despite the fact that the effector pool exhibits minimal basal homeostatic proliferation. The continued survival of these effector-phenotype cells is not simply due to them differentiating into memory cells, because the majority of transferred KLRG-1<sup>hi</sup>IL-7R $\alpha$ <sup>lo</sup> cells maintain this phenotype for many weeks after adoptive transfer (Sarkar et al., 2008). Likewise, studies on Sendai infected mice suggest CD8<sup>+</sup> T cells with effector-like properties (including sustained expression of Granzyme B expression) persist for many months after infection (Hikono et al., 2007).

A more extreme situation is where repeated immunizations have been used to boost the memory T cell pool and for populations in non-lymphoid sites. Compared to primary immune responses, the boosted CD8<sup>+</sup> T cell population sustains a population of cells with more effector-like properties (Granzyme B<sup>+</sup>KLRG-1<sup>hi</sup> and, in some cases, IL-7R<sup>lo</sup>) suggesting such cells are not always short lived (Jabbari and Harty, 2006; Masopust et al., 2006a; Masopust et al., 2006b).

This finding leads to the important question of whether such “not-so-short-lived” effector cells have any functional relevance, or are purely detritus waiting to be cleared from tissues? We will return to this question under “function of the long-lived pool” later.

### The persistence of memory

The typical view of a “useful” primed cell is one which is both long-lived (at the population level) and displays the functional traits of a memory cell (including the ability to undergo recall proliferation and differentiation into secondary effector and memory cells). A long-standing controversy regarded whether T cell memory maintenance depended on constitutive stimulation by cognate antigen. Resolution of this argument had implications for whether

vaccines should provide persistent depots of antigen or require periodic boosting. In contrast to naïve T cells, memory CD8<sup>+</sup> and CD4<sup>+</sup> T cells are maintained in the absence of MHC molecules (Freitas and Rocha, 2000; Murali-Krishna et al., 1999; Swain et al., 1999). Memory CD8<sup>+</sup> T cells appear to maintain both self-renewal and function in the complete absence of MHC I, (Leignadier et al., 2008; Murali-Krishna et al., 1999) however, optimum memory CD4<sup>+</sup> T cell function may depend on exposure to MHC II (De Riva et al., 2007; Kassiotis et al., 2002). Most recently, it was elegantly demonstrated that ablation of TCR also had no effect on memory CD8<sup>+</sup> T cell survival (Leignadier et al., 2008). However, chronic infections can markedly alter T cell survival requirements, resulting in cells that are “addicted” to antigen and wane upon transfer to naïve hosts (Shin et al., 2007).

After clearance of acute infections, memory CD4<sup>+</sup> and CD8<sup>+</sup> T cell maintenance depends on IL-7 and IL-15, which together support survival and basal homeostatic proliferation of the pool (for a recent comprehensive review of this interesting topic, see (Surh and Sprent, 2008)). Competition for these homeostatic cytokine resources may define a limited carrying capacity of the immune system for memory T cells. Such a mechanism may ensure that exposure to numerous infections throughout life does not result in an ever increasing expansion in the size of memory T cell compartment with age.

Most studies have focused on memory populations sustained in secondary lymphoid organs (SLOs) and parenchymal sites, but recent evidence suggests memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells may make a primary home in the bone marrow (Becker et al., 2005; Mazo et al., 2005; Tokoyoda et al., 2009). This notion creates an interesting anatomic separation issue in the response to non-systemic recall antigen, suggesting an obligation by antigen presenting cell to find the bone marrow resident memory cells, or egress of the memory T cell population to access SLOs or non-lymphoid sites.

It should be noted that Tem cells may express lower amounts of CD122 and undergo less homeostatic proliferation than Tcm cells, which may account for the relatively short life span of Tem cells within blood observed after certain primary infections (Jabbari and Harty, 2006; Wherry et al., 2003). However, heterologous prime-boost vaccination can result in a 4-fold expansion of the Tem cell pool outside of lymph nodes, with little evidence for a reciprocal reduction among the Tcm cell compartment (Vezys et al., 2009). In the absence of further infection, this compartment appears stable, suggesting the size of the Tem cell compartment is not subject to stringent regulation, and may depend on different factors for survival than those defined for Tcm cells. Indeed, in humans, CD45RA<sup>+</sup> Tem cells gradually accumulate with age (Czesnikiewicz-Guzik et al., 2008). This process may occur in the absence of CMV infection, and may be distinct from the memory-like T cell clonal expansions observed among the very old (Clambey et al., 2008; Ely et al., 2007; Messaoudi et al., 2006).

The durability of T cell memory remains unclear. In mice, infection with LCMV results in a stable memory CD8<sup>+</sup> T cell pool for >900 days, whereas memory CD4<sup>+</sup> T cells underwent a 50-fold attrition over the same period (Homann et al., 2001). Although human memory T cell longevity studies are in their infancy (due to their greater logistical complications), a few important studies have examined this issue in situations where periodic pathogen re-exposure are unlikely. Fairly robust levels of memory CD8<sup>+</sup> T cells were detected >15 years after infection with Puumala virus (PUUV) without evidence for viral persistence or re-infection (Van Epps et al., 2002). Up to 1% of CD8<sup>+</sup> and CD4<sup>+</sup> T cells remained specific for measles virus among adults that had a history of natural childhood infection (Nanan et al., 2000). The most comprehensive study to date recounts a non-randomized, cross-sectional analysis of CD4<sup>+</sup> and CD8<sup>+</sup> T cell memory performed between 1 month and 75 years after smallpox vaccination (Hammarlund et al., 2003). Virus-specific memory T cells were detectable up to 75 years after a single vaccination, with an estimated half-life between 8 and 15 years among

both CD8<sup>+</sup> and CD4<sup>+</sup> T cells. However, it should be noted that long-lived CD8<sup>+</sup> T cell immunity was not detected in all individuals, although the reasons for this are unclear.

Although these observations support the hypothesis that T cell memory may persist for the life of the host, it has been shown that certain heterologous infections can result in the ablation of pre-existing memory CD8<sup>+</sup> T cells. This process is unlikely to be explained by direct competition between memory cells as the extent of attrition was severe (25-90%), and attrition was induced within only a few days of infection via a Type I or II interferon-dependent process (Dudani et al., 2008; Selin et al., 1999). It will be important to determine how natural infections, as well as immunization, impact the pre-existing memory T cell pool in humans. Although information remains limited, a recent study failed to detect an impact on the number of blood-born influenza and EBV specific CD8<sup>+</sup> T cells upon primary HCMV infection (van Leeuwen et al., 2006). Although more studies are needed, available data suggest that T cell memory in humans is generally long-lived, but perhaps not as durable as humoral immunity, which has estimated half-lives that may exceed 50 years (Amanna et al., 2007; Crotty et al., 2003). However, it should be stressed that these data are derived from individuals whom were exposed to live-replicating agents. It is possible that certain immunizations may fail to produce truly long-lived memory T cells (see below).

### Non-lymphoid tissues: Getting there

Activation within SLOs precipitates changes in homing molecule expression that results in the anatomic re-distribution of antigen-specific T-cells. Changes in migration potential are likely regulated both by the strength of stimulation, reflected by the intensity and duration of signaling via the TCR, co-stimulation, and the inflammatory milieu, and also by location specific cues that vary within different SLO environments.

In situations where strength of stimulation is likely reduced, either via high dose adoptive transfer of transgenic CD8<sup>+</sup> T cells, or by spiking in naïve T cells a few days after the initiation of infection (artificially introducing “latecomers”), a much higher proportion of memory T cells express lymph node homing molecules (Catron et al., 2006; D'Souza and Hedrick, 2006; Sarkar et al., 2007; van Faassen et al., 2005). Similarly, CD4<sup>+</sup> T-cells within lung were found to have undergone a larger number of divisions than their counterparts within lymph nodes (Roman et al., 2002). Interestingly, “strength of stimulation” may be cumulative, as boosting memory CD8<sup>+</sup> T-cells via repeated infection results in the preferential accumulation of CD62L<sup>-</sup> cells that populate non-lymphoid organs (Jabbari and Harty, 2006; Masopust et al., 2006a).

Location specific cues have been best defined within SLOs associated with the intestinal mucosa and the skin (Agace, 2006; Mora et al., 2008; Sigmundsdottir and Butcher, 2008). After activation within Peyer's patches and mesenteric lymph nodes, activated T cells preferentially up-regulate receptors involved in homing to the small intestine. This instructional program is delineated in part by CD103<sup>+</sup> DCs and the local availability of retinoic acid. In contrast, T-cells primed within the skin-associated inguinal lymph nodes preferentially up-regulate skin homing receptors, a process intriguingly promoted by local higher concentrations of Vitamin D3 metabolites. Although priming within local SLO environments thus favors T cell trafficking to regionally-associated non-lymphoid tissues, it should be stressed that there are several examples in which local immunization routes result in widely disseminated T cell responses within all tissues examined (Kaufman et al., 2008; Liu et al., 2006; Masopust et al., 2004). It remains possible that promiscuous seeding of numerous non-lymphoid compartments is dependent on immunization regimens that induce strong “strength of signal” priming, as induced by live replicating agents, and that subunit vaccines may result in responses that remain preferentially distributed within lymph nodes and local tissues.



## Non-lymphoid tissues: Upon arrival

After clearance of infection, large numbers of T cells remain distributed within non-lymphoid tissues (Masopust et al., 2001b; Reinhardt et al., 2001). Confirming predictions based on analyses of human blood T cells that lacked lymph node homing receptors (Sallusto et al., 1999), T cells isolated from tissues differed from “classic” memory T cells in many important functional respects. This precipitated the paradigm that long-lived (i.e. “memory”) T-cells should be divided into two subsets; Tcm cells, which exhibit classic memory T cell properties and traffic through SLOs, and Tem cells, which re-circulate through non-lymphoid tissues (Sallusto et al., 2004).

The generic term “non-lymphoid tissue” accounts for all cells outside of the major inductive sites of immune responses (e.g. lymph nodes and the white pulp of spleen) and does not discriminate between the varied anatomic compartmentalization outside of SLOs. For instance, some cells are likely confined to the vasculature, even among solid organs that have been perfused to remove the majority of red blood cells. One example includes the liver, in which the vast majority of lymphocytes are likely confined to the sinusoids, rather than being located within the parenchyma (Geissmann et al., 2005). Some of these cells may be just quickly passing through, and others may be actively retained on endothelium as has been recently described for a subset of monocytes (Auffray et al., 2007). In contrast, memory T cells are also present within the parenchyma of non-lymphoid tissues, such as the skin and mucosal tissues. Moreover, there are populations of non-lymphoid T cells, such as those within the lung airways, that are localized neither in blood vessels nor in the tissue parenchyma (Woodland and Kohlmeier, 2009). To add to this complexity, there are regional specializations in environments with respect to the cytokine milieu, density of lymphocytes and other hematopoietic cells, tissue architecture, and environment. These tissue-specific differences may reflect exposure to microbial products (for example, the commensal flora in the intestinal mucosa), and the ability of each tissue to tolerate inflammation while maintaining organ function (for instance, inflammation in the lower lung airways inhibits gas exchange).

Therefore, it is perhaps not surprising that T cell properties vary considerably among different anatomic locations, and recent data suggest that the tissue environment plays a direct role in shaping this process independent of variables associated with T cell priming within SLOs. For instance, the environment of the peritoneum in mice induces expression of CD49d among memory CD4<sup>+</sup> T cells (Kassiotis and Stockinger, 2004). The lung environment also directly modulates memory CD8<sup>+</sup> T cell phenotype (Marzo et al., 2007). For instance, transfer of Tcm cells into the lung airways via the trachea results in the rapid adoption of a unique tissue-specific signature phenotype characterized by down-regulation of CD27 and CD127 (Kohlmeier et al., 2007). Cells in the lung airways also down-regulate Ly6C and CD11a, which are typically highly expressed among antigen-experienced T cells in other tissues. These cells are not long-lived, but must be continually maintained by an influx of memory T cells from the periphery (Ely et al., 2006). A quite distinct tissue-specific phenotype is adopted among CD8<sup>+</sup> T-cells after entry within the epithelium of the small intestine. These cells adopt several cardinal features of recently activated T-cells, including up-regulation of CD69 and the maintenance of lytic activity indefinitely (Masopust et al., 2006b). Interestingly, these cells express only low amounts CD122 and undergo relatively little homeostatic division, yet the population can remain stable without evidence for continued recruitment, suggesting that they may have unique maintenance requirements (Ma et al., 2009; Masopust et al., 2006b). Intravenous transfer of memory T-cells from the intestinal epithelium, followed by re-stimulation, resulted in a secondary response that distributed to numerous tissues. Those daughter cells that were recovered from spleen eight months later had adopted the cardinal features of classic Tcm cells, further supporting the hypothesis that tissue environments directly influence T-cell differentiation state, and also indicating that a very “effector-like” phenotype is not terminal

(Masopust et al., 2006b). Although we only cited a few examples, it appears likely that T-cells in many tissues, including skin and the central nervous system, may adopt tissue-specific signature phenotypes (Gebhardt et al., 2009; van der Most et al., 2003). Non-lymphoid memory T-cells that are essentially occupying blood vessels or are rapidly re-circulating, such as those in the liver or low pressure alveolar capillary beds of the lung, may be less subject to tissue derived environmental cues.

In this light, conceptually lumping together all non-lymphoid sites is not likely to account for the complexity of T-cell differentiation states. Experimentally, cells isolated from a single non-lymphoid tissue are unlikely to represent the properties of Tem cells in other tissues. This issue is but one reflection of the difficulty in defining Tem cells as a homogenous entity. In fact, in its current usage, Tem cells reflect a broad array of T cell differentiation states that differ from the classical definition of memory T cells (Figure 2). These differentiation states are regulated both by the strength of stimulation during priming, but also by largely unknown tissue-specific factors. An important issue of continuing study is whether memory cell survival is equivalent in all tissue locations (Woodland and Kohlmeier, 2009), although the challenge will be to assess this in situ. It must also be appreciated that properties of memory T cells may be affected transiently or permanently by local cues. These observations are not an attempt to muddy the waters, but rather to confront the true diversity of T-cell qualities and to acknowledge the rather imprecise meaning of the term Tem cells. One current challenge is to determine more completely how T cell differentiation states are regulated, how they are coupled with T cell trafficking, and how these issues relate to protective immunity (see below).

### **T cell protective Immunity: a matter of quality, quantity, time and location**

This review has recounted numerous studies that have attempted to penetrate the processes by which memory T-cells are made, and the factors that govern their maintenance, anatomic distribution, and functional potential. An overarching *raison d'être* of all these studies is to determine how to exploit this knowledge for the development of safe, effective vaccines. Achieving this ultimate goal will require a thorough understanding of the parameters that relate to protection.

For a memory T cell to participate in protection, it must still be present upon re-infection. As discussed above, certain subsets of antigen specific T cells wane at a leisurely rate after clearance of infection. However, does this mean that these short-lived memories (or long-lived effectors) are useless by-products of an immune response, or do they confer a transient state of heightened host protection? In a mouse model of LCMV infection, CD62L<sup>-</sup> CD8<sup>+</sup> T cells gradually decay or convert to central memory T cells (Wherry et al., 2003). Although these cells were somewhat impaired in their ability to undergo proliferation upon re-stimulation, they were able to confer protection upon transfer to secondary recipients. A more natural, albeit anecdotal, example comes from a mouse model of heterosubtypic immunity (Liang et al., 1994). Mice were infected with influenza virus, resulting in long-lived memory in most tissues. As described above, short-lived T-cells persist in the lung airways, but maintenance of this population depends on continued recruitment from the periphery. For reasons unknown, this recruitment abates over time, resulting in the presence of a very small population of antigen-specific CD8<sup>+</sup> T-cells by six months after infection. Gerhard and colleagues found that protective immunity against a serotypically distinct influenza virus (against which T-cells mediate protection) waned with a kinetics similar to the attrition of the memory population in the lung airways (Liang et al., 1994).

This finding suggests that short-lived T-cells within the lung airways provide short-lived protection, and also supports the appealing notion that T-cells positioned at the point of pathogen entry may play an important role in protective immunity against certain infections.

Woodland and colleagues re-visited this issue more directly by transferring Sendai virus specific T-cells from the lung airways of immune mice into the airways of naïve mice (Hogan et al., 2001). In this model, they showed that CD4<sup>+</sup> T-cells conferred some degree of protection upon homologous challenge. Similar findings were recently reported in an elegant model of herpes simplex viral challenge, demonstrating a role for skin resident CD8<sup>+</sup> T cells in mediating rapid protection at the site of infection (Gebhardt et al., 2009). These studies support concerted efforts to establish site-specific T-cell immunity through vaccination in the hopes of contributing to rapid containment of pathogens such as HIV. However, defining the role that T-cells positioned outside of SLOs play in protective immunity remains a major challenge.

Delineation of memory T-cells into Tcm and Tem cell subsets has attracted substantial interest among vaccinologists, in the hopes that creation of the right “kind” of memory T-cells might provide the key to protection. In turn, so-called Tcm and Tem cells have been compared in various protection models, with conflicting results, depending on the experimental system. It should be noted that Tcm cells are often generated by different methods among different studies (for example, live viral infections vs. heat killed *Listeria monocytogenes*, LM), and delineated solely by the expression of markers such as CD127 and CD62L. Such a readout does not account for variables in the function of Tcm cells after different priming regimens (e.g. whether they rapidly produce IFN- $\gamma$  upon re-stimulation). Similar concerns regard comparisons of Tem cells among different studies, highlighting the challenges that imprecise definitions of T-cell subsets pose to the field. With these substantial caveats in mind, it appears that Tem cells are most effective at controlling vaccinia virus and LM infections, as measured by evaluating infectious burden 3-4 days after infection (Bachmann et al., 2005; Huster et al., 2006). In contrast, Tcm cells may be more effective at controlling a systemic challenge with a strain of LCMV that causes a chronic infection in naïve mice (Wherry et al., 2003). In the latter study, it should be noted that Tcm cells did not contribute to protection until more than a week after viral challenge. In this instance, it seems likely that substantial expansion of the antigen-specific CD8<sup>+</sup> T cell population, as well as differentiation into effector cells, was required before it was able to contribute to viral control. A tentative conclusion that could be drawn from these studies is that Tem cells might be more effective at controlling infections very early, especially outside of SLOs. However, if the infectious challenge exceeds the capacity of pre-existing Ag-specific cells to control infection, then the greater proliferation potential of “classic” memory CD8<sup>+</sup> T-cells wins the day. Thus, the importance of different subsets may vary depending on the pathogen in question, the route and dose of infection, and the quantity of antigen-specific T cells present prior to infection.

The above discussion raises another important question. How important is T cell quantity? This question takes on additional prescience in light of the recent and highly publicized failure of the Phase 2b efficacy STEP trial, which represented an ambitious test of CD8 T cell vaccination against HIV in humans. Although this vaccine generated 100-500 HIV-specific memory CD8<sup>+</sup> T cells per 10<sup>6</sup> PBMC, it afforded no protection. Was this a failure of CD8<sup>+</sup> T cell quantity, quality and/or anatomic distribution, or rather a revelation that CD8<sup>+</sup> T cells cannot protect against HIV (Masopust, 2009)? There is little information to guide informed speculation. However, recent studies found that higher frequencies of CD8<sup>+</sup> T cells (~2000 per 10<sup>6</sup> PBMC) generated by prime-boost vaccination or immunization with a persistent vector, afforded some degree of protection against fairly stringent SIV challenges in non-human primates (Hansen et al., 2009; Liu et al., 2008). Although this bolsters hope in the CD8 T cell vaccine concept, more information is needed regarding the relationship between CD8<sup>+</sup> T-cell quantity, quality, and protective immunity. In this light, a recent study from Harty and colleagues carries both good and bad news. Using a creative model of prime-boost vaccination, they modulated the number of memory CD8<sup>+</sup> T cells specific for Plasmodium (Schmidt et al., 2008). They found that CTL immunity was sufficient to prevent blood-stage parasitemia, but only if the quantity of antigen specific CD8<sup>+</sup> T-cells exceeded a very large minimum threshold

(>1% of blood lymphocytes). In comparison, 100-1000 fold fewer CD8<sup>+</sup> T cells were sufficient for protection against LM or LCMV. Although this study did not rule out changes in quality induced by the different immunization schemes that may also have contributed to protection, it illustrates that the requirements for CD8<sup>+</sup> T-cell-mediated protective immunity against some pathogens may greatly exceed that generated by current human vaccine strategies that rely heavily on replication-deficient vectors.

## Bad Memories

Just as there are many ways to define a “good” memory cell, there are also ways these cells can fail to contribute to the recall response. A well studied case is CD8<sup>+</sup> T memory cells primed in the absence of CD4<sup>+</sup> T cell help. In many acute infection models, the production of effector and memory CD8<sup>+</sup> T cell populations follows the normal pattern despite CD4<sup>+</sup> T cell deficiency. These “unhelped” CD8<sup>+</sup> T memory cells show reduced maintenance, but more dramatically show impaired function (including protection) and an almost complete loss of recall proliferative capacity (Janssen et al., 2003; Sun and Bevan, 2003) (Janssen et al., 2003; Shedlock and Shen, 2003). Although the exact basis for these defects is currently unclear, an unexpected twist is that T-bet deficiency partially rescues the “unhelped” CD8<sup>+</sup> T memory cell phenotype (Intlekofer et al., 2007). Interestingly, analogous defects in recall proliferative potential are observed for CD8<sup>+</sup> T cells which lack expression of IL-2Ra (CD25) (Williams et al., 2006), raising the possibility that CD4<sup>+</sup> T cell-produced IL-2 may be an critical element in CD4<sup>+</sup> T cell “help” for CD8<sup>+</sup> T cell memory. Thus, despite the fact that both unhelped and CD25-deficient (*Il2ra*<sup>-/-</sup>) CD8<sup>+</sup> T memory cells resemble Tcm cells, they show defective homeostasis and/or reactivity (Fig 3).

Functional deficiency may also arise due to the suboptimal priming, and this may be difficult to observe by simply monitoring T cell numbers or individual functional readouts. Testing various vaccination approaches for priming CD4<sup>+</sup> T cell responses to Leishmania antigens revealed that protective immunity correlated with the cells capacity for multiple functional responses (Darrah et al., 2007).

## Alternative ways to make memory cells: Heterologous memory

Are the specificities of T cell memories always restricted to the pathogens that induced them? Perhaps not. One important example is the case of heterosubtypic immunity, by which memory CD8<sup>+</sup> T cells mount secondary responses upon challenge with serologically distinct, but phylogenetically related viruses. Such observations have been made in several models, including infection of mice with vesicular stomatitis viruses of the New Jersey and Indiana strains, and influenza viruses, which also confer a degree of protective immunity against the second challenge (Christensen et al., 2000). In addition, situations have also been documented in which immunity to one virus, LCMV, confers protection to an unrelated virus, vaccinia. Interestingly, the specificity of cross-reactive CD8<sup>+</sup> T cell clones between LCMV and vaccinia virus varies among individual inbred mice, suggesting that cross-reactivity between different infectious agents may not be uncommon (Kim et al., 2005). Indeed, cross-reactive human CD8<sup>+</sup> T cell clones have been detected that recognize epitopes from both influenza A virus and Epstein-Barr virus (Clute et al., 2005). These observations suggest that primary responses among individuals with a diverse repertoire of memory CD8<sup>+</sup> T cells may contain re-activated secondary clonal expansions of rare cross-reactive pre-existing memory T cells. Such events may influence the quality and immunodominance hierarchy of the response, and may have implications for protective immunity.

## “Homeostatic” memory

Over the last 10 years it has become clear that the scheme shown in Fig 1 is not the only means to generate memory T cells. During studies on T cell homeostasis, it was found that naïve cells respond to T cell lymphopenia by undergoing a slow proliferation (variously called “homeostatic proliferation” or, less ambiguously “lymphopenia induced proliferation”, or LIP), during which time the naïve cells change in their phenotype and function to resemble memory cells (Surh and Sprent, 2008). This process involves recognition of self peptide-MHC molecules and the cytokines IL-7 and IL-15 (Surh and Sprent, 2008), but cells responding to lymphopenia appear not to transition through a detectable effector stage. This process may therefore resemble the low intensity stimuli which promote memory T cell differentiation discussed earlier. LIP generates memory cells which exhibit functional properties similar to “true” (i.e. antigen primed) memory cells (Surh and Sprent, 2008), and are capable of undergoing a strong proliferative response when stimulated by foreign antigen and mediating protective immunity against infection (Hamilton et al., 2006). Although typically studied in artificial situations of lymphopenia, similar process may occur in normal physiological stages, such as during the lymphopenia associated with neonatal mice (Ichii et al., 2002; Min et al., 2003; Schuler et al., 2004). This raises the possibility that the endogenous T cell pool may contain memory-like cells produced without conventional priming

While an “endogenous” memory T cell pool has long been documented in mice (including those maintained in SPF or even germ free conditions (Haluszczak et al., 2009; Huang et al., 2005), the specificity of this pool was largely unexplored. Recent studies suggest that the endogenous pool of memory phenotype cells includes cells with reactivity toward unencountered foreign antigens (Haluszczak et al., 2009). This normal pool, which has hallmarks of deriving from LIP, exhibits at least some of the functional traits of memory cells (Haluszczak et al., 2009). However, this population is extremely rare, representing only a fraction of the naïve antigen specific precursor pool, and how these cells contribute to physiological immune responses, and whether their contribution differs from that of their naïve counterparts, awaits further studies. However, these findings do suggest unprimed endogenous memory cells may be active participants in “primary” immune responses.

## Concluding remarks

The chief objectives in this review were not only to discuss new developments in our understanding of the generation and function of memory T cells, but to highlight the fact that memory T cell populations are tremendously diverse in terms of phenotype, function, developmental plasticity, distribution, longevity and protective capacity. A single, or even a handful, of markers is unlikely to reliably predict either the developmental or protective potential of “subsets” that transcends single experimental systems. Ignoring this complexity, and splitting T cells into poorly defined effector and memory subsets, may create disagreements that are more grounded in semantics than biology. However, a range of innovative techniques, including analyses of single cells, cellular barcoding, and genome wide transcriptional profiling, seem poised to push our insight even further into T-cell fate decisions. When such information is coupled to bona fide studies of protective immunity, the field has the potential to make tremendous and much needed contributions to the development of protective T-cell vaccines.

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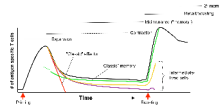


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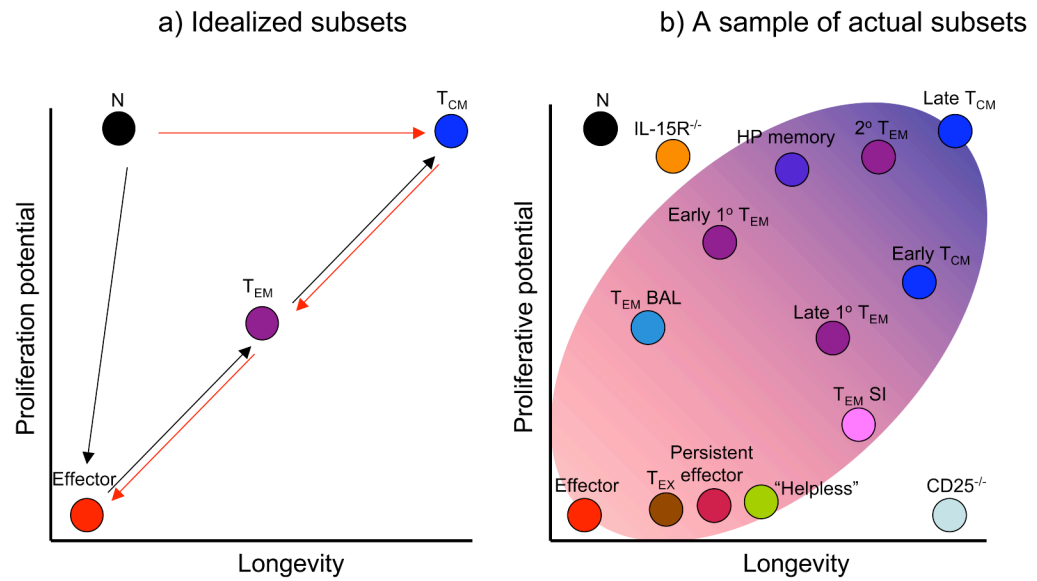
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**Figure 1. Making lasting memories**

The schematic shows numbers of antigen specific T cells (black line) at various stages after priming and boosting of a prototypical acute immune response. During the primary response, the fate of typical effector (red line) and memory (green line) cells is shown. Also shown are populations of intermediate longevity whom may also contribute to protection from re-infection (maroon and gold lines).

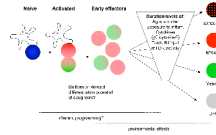


### c) Common subsetting parameters

Proliferation potential
Cytolytic activity
IFN- $\gamma$ /TNF $\alpha$ production capacity
Turnover
CD62L/CCR7 phenotype
IL-2 production capacity
Longevity

**Figure 2. Embracing diversity**

(left) The chart shows the relative longevity and recall expansion potential of “idealized” naïve, effector, Tcm and Tem cell populations, as they are typically discussed, as well as conflicting models of lineage differentiation. (right) The same chart is shown with inclusion of some populations found during actual immune responses. The shaded area indicates a distribution “cloud” of cells with effector-through-memory like properties. Many populations in this cloud would typically be designated “Tem” but, as indicated, there is also diversity among the “Tcm” cell pool. Shown are memory populations identified in various tissue sites, including Tem cells from lung bronchoalveolar lavage (BAL) (Ely et al., 2003) and small intestine (SI) (Masopust et al., 2006b) and Tem and Tcm cells isolated from spleen at different memory time points (“Early” and “Late” Tcm and Tem) (Roberts et al., 2005). Also shown are CD8<sup>+</sup> T cells primed without sufficient CD4<sup>+</sup> T cell help (“Helpless”), memory-like cells made through lymphopenia-driven homeostatic proliferation (“HP” memory cells), T cells driven to “exhaustion” by chronic antigen exposure (“Tex”) and effector cells maintained into the long-lived phase (“Persistent effector”) (references provided in the main text). The position of CD8<sup>+</sup> memory T cells produced in IL-15- (or IL-15Ra-) deficient mice, and of CD25-deficient CD8<sup>+</sup> memory T cells is also illustrated, though it is not yet clear whether such cells are representative of populations generated in natural responses (hence these cells fall outside the “cloud”). Relative positions of populations are not intended to be precise, but merely to indicate the diversity of groups identified using only these two parameters. The box indicates further layers of complexity would be revealed if additional functional parameters (some of which are listed here) were included in the subset definitions.



### Figure 3. Walking the tightrope: factors affecting differentiation of activated T cells

After stimulation of naïve T cells by antigen bearing APC, the first cell division is asymmetric and may produce cells with distinct potential to become effector or memory cells (indicated by shading of daughter cells). However, the products of initial stages of the expansion phase (“Early effectors”) probably all express key effector molecules (including Granzyme B in CD8<sup>+</sup> T cells and IFN- $\gamma$  in CD8<sup>+</sup> T and Th1 cells), yet lack later effector markers. This early effector pool may have full or only limited potential to become any of the differentiated populations shown on the right. The production of effector and memory populations during the remainder of the expansion phase is also conditioned by the duration and intensity of various signals. These include signaling associated with the TCR and also cytokine receptors. Such signals may dictate expression of key transcription factors including T-bet and Blimp-1, and also control the metabolic status of the activated cell (including that regulated by mTOR activity). Unusually strong or persistent stimuli may generate “exhausted” T cells, whereas inadequate stimuli produces “unfit” cells. In between these extremes, effector and memory differentiation may be driven by cumulative cues operating on the early effector cell (or by selectively favoring outgrowth/survival of cells with a bias toward the effector or memory fate).