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Diversity in CD8$^+$ T cell differentiation

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CD8$^+$ T cells are key effector cells of the adaptive immune system, however their activity must be tightly regulated to allow pathogen clearance whilst preventing immunopathology and autoimmunity. In this review, we summarise the diversity of responses that CD8$^+$ T cells make to antigenic stimulation with a focus on how CD8$^+$ T cell responses are regulated to achieve different immune outcomes. In particular, we discuss phenotypic diversity during tolerance induction as well as signals that drive effector and memory cell differentiation in response to infection.

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Introduction

The peripheral CD8$^+$ T cell repertoire is in a constant state of flux as these cells see a myriad of environmental signals that are continuously varying. In the steady state, naïve CD8$^+$ T cells must compete for homeostatic survival signals, such as MHC class I (MHC I) and interleukin-7 (IL-7), in order to survive. Moreover, antigen encounter can synergise with environmental signals to drive CD8$^+$ T cells down numerous differentiation states that vary both in effector function and survival capacity. Correct coordination of these differentiation states is crucial for both the maintenance of peripheral tolerance and the expansion and persistence of pathogen-specific clones. This review aims to summarise the diversity of CD8$^+$ T cell responses to antigen, with a focus on recent work in the fields of CD8$^+$ T cell tolerance, effector cell differentiation and memory (Figure 1).

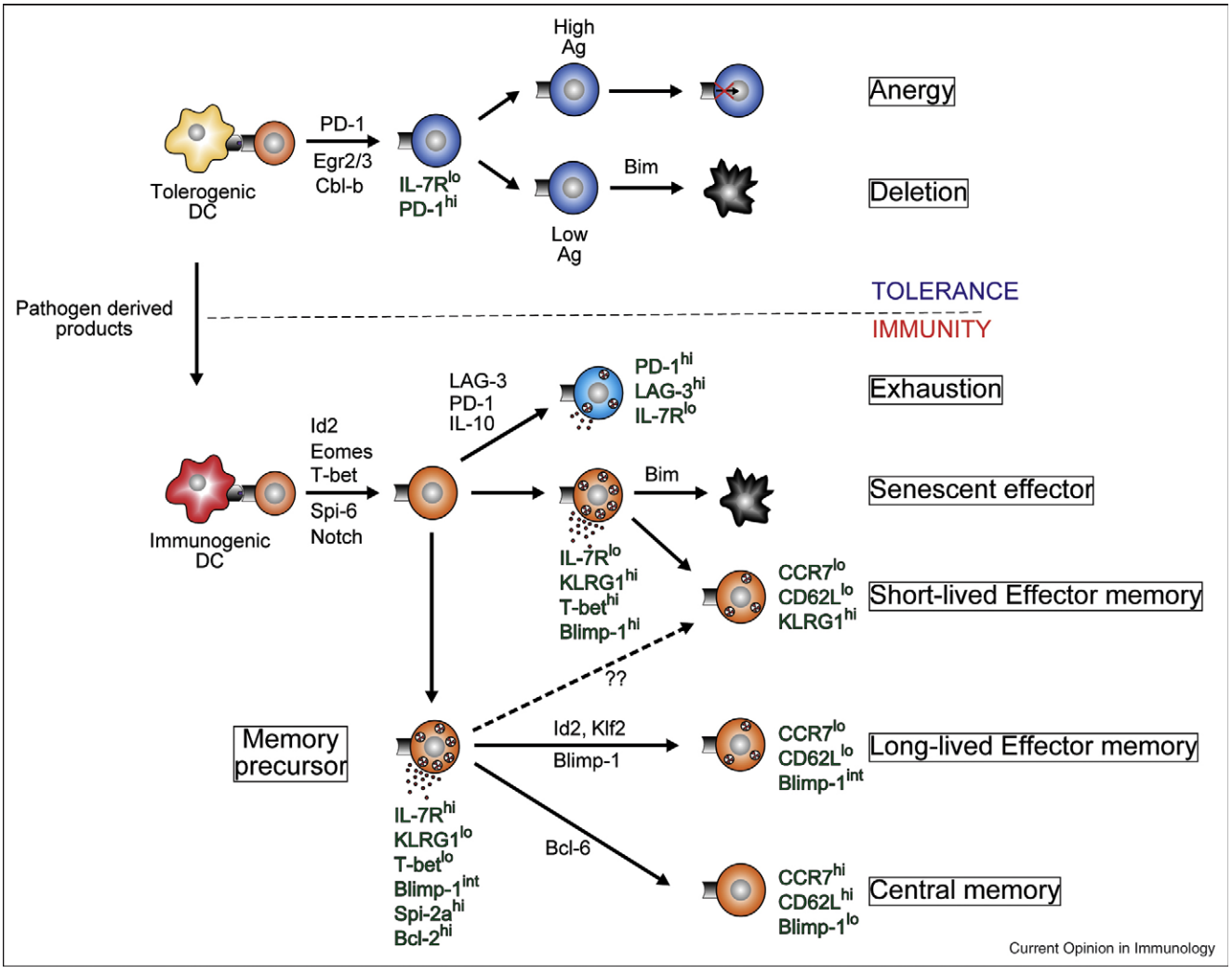
Multiple paths to peripheral tolerance

Whilst autoreactive CD8$^+$ T cells are purged from the reportoire during thymic negative selection, this is not a perfect process, and rogue autoreactive CD8$^+$ T cells are often released into the periphery [1]. Peripheral tolerance mechanisms have thus evolved to purge the repertoire of these dangerous self-reactive cells. Dendritic cells (DCs), a key antigen-presenting cell population, are thought to drive this process by inducing tolerance within any T cells that recognise antigen presented by DCs in the steady state.

Upon steady-state antigen recognition, peripheral tolerance induction in CD8$^+$ T cells can result in two distinct differentiation states: deletion and anergy (Figure 1). In both tolerance fates, CD8$^+$ T cells generally fail to develop effector functions despite proliferating in response to antigen [2,3], although cells can pass through an effector phase *en route* to tolerisation in some models [4,5]. However, despite these similarities, each tolerance fate exhibits unique features. Whilst anergic CD8$^+$ T cells persist in a hyporesponsive state caused by T cell receptor (TCR) signalling defects [3], cells undergoing deletion die by apoptosis triggered by the proapoptotic BH3-only protein Bim [6]. Nevertheless, contraction does occur in models of CD8$^+$ T cell anergy and this death requires Bim (IA Parish and WR Heath, unpublished observations). The biological reason for maintaining anergic cells is currently unclear, but one possibility is that anergic CD8$^+$ T cells adopt an immunoregulatory state [7].

Deletion and anergy are often considered disparate cell fates, however recent data have questioned whether these cell fates are as molecularly distinct as previously thought. One of the features of anergic T cells is upregulation of the transcription factors Egr-2 and Egr-3 by NFAT activation in the absence of AP-1 activation [8,9]. Egr-2 and Egr-3 then drive expression of immunosuppressive E3 ubiquitin ligases, such as Cbl-b, which degrade key signalling components and attenuate TCR signalling capacity [10]. Interestingly, both Egr-2 and Cbl-b (as well numerous other anergy associated genes) were strongly upregulated during deletion [11] suggesting that molecular parallels exist between deletion and anergy. Furthermore, signalling through the inhibitory signalling molecule PD-1 is required for both CD8$^+$ T cell anergy [12] and deletion [13,14]. Finally, at least in CD4$^+$ T cells, blocking death in a model of deletion leads to a fate of anergy [15•]. Although still speculative, it is possible that common genetic pathways direct states of anergy and deletion in tolerised CD8$^+$ T cells, but the outcome is largely dependent on the overall level of TCR signalling. It is known that high antigen levels typically cause anergy whereas lower antigen levels lead to deletion [16•]. Thus, in situations of weak or tissue-restricted antigen presentation, deletion may prevail, because the expression of genes inhibitory to TCR signalling, coupled with lower IL-7 receptor expression [11],

Figure 1



A model for generating diverse CD8$^+$ T cell fates. (Top portion) Naïve CD8$^+$ T cells (brown cell) that encounter antigen on steady state, tolerogenic DCs (beige cell) proliferate without acquiring effector functions (dark blue cells) in a process dependent on PD-1 and the molecules Egr-2, Egr-3 (Egr-2/3) and Cbl-b. Such tolerised T cells typically upregulate PD-1 and downregulate IL-7R as indicated, and either die (black cell) or become anergic (i.e. deficient in TCR signalling as denoted by the red cross). Antigen (Ag) levels control this process, with high Ag levels promoting anergy, and low Ag levels causing deletion. (Lower portion) Immunogenic DCs (red cell), which have encountered pathogen-derived products, activate naïve CD8$^+$ T cells to form effector cells (cytotoxic granules and production of effector cytokines are indicated). This differentiation process is regulated by molecules such as Id2, Spi-6, Notch, T-bet and eomesodermin (Eomes). These effector cells can adopt multiple cell fates, such as memory precursor cell fates (characterised by IL-7R^{hi}, KLRG1^{lo}, T-bet^{lo}, Blimp-1^{int}, Spi-2a^{hi} and Bcl-2^{hi} expression; bottom left) or short-lived effector cell fates (with IL-7R^{lo}, KLRG1^{hi}, T-bet^{hi} and Blimp-1^{hi} expression; middle). Such short-lived cells can either become senescent effectors and die by bim-dependent apoptosis, or persist into early memory as short-lived effector memory cells that are CCR7^{lo}, CD62L^{lo} and KLRG1^{hi}. By contrast, memory precursor cells are long-lived and can be CCR7^{lo}, CD62L^{lo}, Blimp-1^{int} effector memory cells (promoted by Id2, Klf2 and Blimp-1), or CCR7^{hi}, CD62L^{hi}, Blimp-1^{lo} central memory cells (driven by Bcl-6). Evidence exists that KLRG1^{lo} effector cells can also give rise to KLRG1^{hi} short-lived effector memory cells (denoted by broken arrow and question marks; NS Joshi, TW Hand and SMK, unpublished observations). In some chronic viral infections, PD-1, LAG-3 and IL-10 causes cells to acquire an exhausted phenotype (light blue cell) characterised by high PD-1 and LAG-3 expression and low IL-7R levels. Although the exhausted cell is depicted as sharing a common effector cell precursor with other cell fates, it is currently unclear at what point during effector cell differentiation this fate branches.

promote cell death by 'starving' cells of MHC and IL-7 survival signals. By contrast, higher antigen levels or ubiquitous antigen expression may provide a sufficiently strong and persistent TCR signal to permit survival of tolerised CD8$^+$ T cells.

Breaking tolerance: the road to an effector cell

Whereas steady-state presentation of antigen by DCs causes CD8$^+$ T cell tolerance by default, antigen presentation in the context of infection drives clonal expansion and CD8$^+$ T cell effector and memory formation.

Effector CD8$^+$ T cells (often termed cytotoxic T lymphocytes, or CTL) can kill antigen-bearing target cells using granzymes and perforin, and can rapidly produce anti-viral cytokines, such as IFN-γ and TNF-α upon TCR ligation [17]. Effector cell differentiation occurs during infection partly because DC recognition of pathogen-derived products creates immunogenic DCs that bear increased amounts of co-stimulatory molecules and antigen–MHC complexes. Antigen encounter on immunogenic antigen-presenting cells for brief periods *in vitro* (as little as one day) can induce CD8$^+$ T cell proliferation and differentiation into effectors, although longer durations of antigen encounter *in vivo* augment clonal expansion [17,18]. Initial *in vitro* studies also suggested that TCR avidity for antigen regulates effector cell differentiation [19], however recent *in vivo* data suggest that, whilst avidity regulates the magnitude of the response, effector (and memory) CD8$^+$ T cells can still form in response to low avidity interactions [20•]. In addition, pathogen activated DCs also upregulate Notch ligands, which can ligate Notch on CD8$^+$ T cells and directly induce cytotoxicity [21•].

Another key set of signals in effector cell formation during infection are innate immune system-derived inflammatory cytokines. In particular, IL-12 and the type 1 interferons (IFNα/β) promote CTL expansion and differentiation *in vitro* [22] and *in vivo* during infection [23,24], in part through induction of cytotoxic molecules and IFN-γ production [22,25]. As different infections will elicit disparate cytokine responses, the cytokines required for CTL expansion and differentiation may vary between infections. For example, CTL responses to *Listeria* infection are more IL-12 dependent [26,27], whilst the CTL response to LCMV infection is more IFNα/β dependent [23,28••]. It should be noted that IL-12 stimulation is most effective in driving CTL differentiation when received simultaneously with a TCR stimulus [26,29], suggesting that, like CD4$^+$ T cells [30], antigenic and cytokine signals must be co-delivered for optimal CTL differentiation. There is additional evidence that other inflammatory cytokines influence CTL differentiation, a topic recently reviewed in detail [22,31].

A number of transcription factors have been identified that promote CTL differentiation. The T-box transcription factor eomesodermin drives acquisition of cytotoxicity and anti-viral cytokine production [32••], although the pathways responsible for eomesodermin induction are currently unclear. Another T-box transcription factor, T-bet, also appears to be a crucial regulator of effector cell differentiation as T-bet deficient CD8$^+$ T cells exhibit diminished cytotoxicity and altered cytokine production [33••]. Moreover, IL-12 induces expression of T-bet [34•,35] providing a mechanism for IL-12 driven CTL differentiation. Nevertheless, IL-12 and IFNα/β can also trigger IFN-γ production via STAT4 activation [25]. The presence of both T-bet and eomesodermin is crucial for normal CTL differentiation as CD8$^+$ T cells deficient in both of these factors develop an aberrant effector phenotype characterised by excessive IL-17 production [36•]. In addition, the transcription factors RBP-J and CREB1 are responsible for the expression of cytotoxic molecules downstream of Notch signalling [21•]. Repressors of effector function also exist; the transcriptional repressor Bcl-6 can repress expression of the cytotoxic molecule granzyme B [37], although Bcl-6 may be more important in controlling memory formation than initial effector cell differentiation (see next section). A major challenge in the field is to determine how this transcriptional network is organised to control effector cell formation and maintain effector molecule gene expression in memory cells in the absence of infection.

Whilst the expression of cytotoxic products in CTLs aids pathogen clearance, they may also be detrimental to the lifespan of a CTL. For example, CTLs lacking the serine protease inhibitors Spi-6 or Spi-2a, which block granzyme B and cathepsin B activity, respectively, show reduced expansion during viral infection [38,39]. Interestingly, Spi-6 is a gene upregulated by the transcription factor Id2, and Id2−/− CD8$^+$ T cells demonstrate a similar propensity to die and decreased clonal expansion during viral infection [40].

It should be noted that during certain chronic viral infections, CD8$^+$ T cells exhibit dysfunction in their ability to mobilise effector functions, such as IFN-γ, TNF-α, IL-2 and cytotoxicity. This dysfunction (often referred to as 'exhaustion') may also be considered a unique CD8$^+$ T cell differentiation state. A network of inhibitory receptors, including PD-1 [41••] and LAG-3 [42], are upregulated on exhausted T cells and appear important in driving the exhausted state. In addition, augmented IL-10 production during chronic infection also promotes an exhausted CD8$^+$ T cell state [43,44] and may operate in a pathway independent of PD-1 [45]. A number of transcription factors are selectively upregulated in exhausted CD8$^+$ T cells (such as PBX3 and Blimp-1) [46] suggesting this effector cell fate is an active process directed by gene regulatory changes.

Life after death: persistence of CD8$^+$ T cells into memory

At the completion of a CD8$^+$ T cell immune response, the majority of effector cells die by Bim-dependent apoptosis [47,48]. However, a proportion of cells survive this contraction process and persist as a memory T cell population that confers long-term protection against reinfection. Memory T cells, although heterogeneous in phenotype, possess a well-defined set of characteristics: they exhibit enhanced proliferative capacity (relative to effector cells), augmented re-expression of

effector genes upon rechallenge (relative to naïve cells) and a unique ability to self-renew and survive for long-periods of time. The molecular basis of many of these features is now emerging. Memory cells exhibit greater proliferative potential relative to effector cells because of reduced expression of the cell cycle inhibitor $p27^{kip}$, increased activity of the kinase CDK6 and reduced expression of the anti-proliferative transcription factor Bmi1 [49,50]. Augmented effector gene re-expression in memory cells appears due to chromatin alterations at effector gene loci [51•,52•]. Finally, unlike naïve or effector cells, long-lived memory $CD8^+$ T cells express receptors for both IL-7 (which promotes survival) and IL-15 (which drives antigen-independent proliferation and self-renewal) [17] leading to long-term maintenance of the memory population. In addition, memory cells express higher levels of factors, such as Bcl-2 and Spi-2a, that both enhance memory cell formation and survival [38,53].

Cells with memory potential are evident early during the immune response, although they may not necessarily display all the hallmark qualities of a mature memory $CD8^+$ T cell [54]. At the peak of the T cell response to certain acute infections, such as LCMV and *Listeria*, these memory precursor cells represent a small proportion of the effector cell population, and can be distinguished by higher IL-7 receptor (IL-7R) expression and lower expression of the senescence marker KLRG1 [34•,55,56]. These IL-$7R^{hi}$ $KLRG1^{lo}$ effector $CD8^+$ T cells demonstrate the highest rate of long-term survival, homeostatic turnover (via IL-15) and secondary responses to reinfection [34•,55–57]. By contrast, most of the remaining effector $CD8^+$ T cells are IL-$7R^{lo}$, express KLRG1, and do not persist or proliferate well following reinfection [34•,56,57]. A population of $KLRG1^{hi}$ IL-$7R^{hi}$ memory $CD8^+$ T cells can additionally be found, although these cells also display a finite lifespan [34•,58].

How do effector $CD8^+$ T cells with memory cell potential form during infection? Our past work suggested that a gradient of T-bet may be established in effector $CD8^+$ T cells, and that high T-bet levels promote terminal effector cell differentiation and senescence, whilst low levels of T-bet permit memory precursor development [34•]. The appropriate balance of T-bet expression appears directly tied to effector cell survival because low levels of T-bet are sufficient for IL-15R/IL-2Rβ (CD122) expression, but high levels of T-bet repress IL-7Rα [34•,57,59•]. Collectively, this suggests that cells with intermediate T-bet levels will have optimal responses to both IL-7 and IL-15, the primary cytokines that sustain memory $CD8^+$ T cells [60]. Recently, it was also found that $CD8^+$ T cells undergo asymmetric cell divisions during the initial cell division [61••] and can be routed down either short-lived or long-lived fates. Perhaps T-bet and other cell fate determining factors are unequally partitioned during these cell divisions, leading to different cell fates. However, other studies have suggested that activated $CD8^+$ T cells are not necessarily committed to one or the other fate so quickly, as longer durations of infection promote terminal differentiation of CTLs [34•,56,62] and IL-12 can augment T-bet expression [34•,35]. Overall, this suggests that environmental signals may amplify or refine the effector cell diversity generated by asymmetric cell division. Establishing a gradient of a lineage-determining transcription factor (such as T-bet) through infection-inducible cytokine levels does not restrict effector T cells to simply two cell fates, but instead permits the broad spectrum of effector and memory differentiation states known to exist. Furthermore, it provides a model with built-in flexibility, whereby the innate immune system can control the magnitude and potency of the effector cell response during diverse infections (which can vary in tropism, intensity and duration). Blimp-1 likely also acts in a graded fashion [57] (RL Rutishauser and SMK, unpublished data), however it is presently unclear if other transcription factors act similarly.

Other factors can promote $CD8^+$ T cell memory formation. The co-stimulatory molecule receptors 4-1BB, OX40 and CD27 can augment memory cell differentiation and survival [17,63]. It is also well established that help from $CD4^+$ T cells is almost always required during the primary response for optimal memory $CD8^+$ T cell formation [17]. Whilst it is unclear what factor $CD4^+$ T cells supply to $CD8^+$ T cells, IL-2 is one candidate as it plays a key role in promoting memory T cell recall responses to secondary infection [64•] and activated $CD4^+$ T cells are an abundant source of this cytokine. In addition, $CD4^+$ T cells may 'condition' DCs to better induce memory formation [65]. Regardless of the mechanism, $CD4^+$ T cell help appears to prevent the memory precursor population from undergoing terminal differentiation; 'unhelped' cells display a higher proportion of T-bet^{hi} $KLRG1^{hi}$ cells and T-bet deficiency can partially restore their function [57].

The memory $CD8^+$ T cell population that eventually forms upon pathogen clearance is heterogeneous and evolves over time. Memory cells are often subdivided based on expression of the lymph-node homing receptors CD62L and CCR7 into $CD62L^{lo}$ $CCR7^{lo}$ non-lymphoid tissue resident effector memory cells (T_{EM}) and $CD62L^{hi}$ $CCR7^{hi}$ lymphoid tissue resident central memory cells (T_{CM}). In studies in mice, most T_{EM} and T_{CM} stem from IL-$7R^{hi}$ memory precursor cells [55,66]. However, some $KLRG^{hi}$ senescent effector cells can persist into early memory as 'short-lived T_{EM}' [34•,55,56] (Figure 1) generating further heterogeneity within the memory population. Whilst $CD8^+$ T_{CM} and T_{EM} both produce IFN-γ, T_{EM} generally display immediate cytotoxicity whereas T_{CM} typically produce more IL-2 and possess a higher proliferative potential [67,68••,69]. Importantly, the antigen-specific memory T cell population composition

changes over time, with T_{EM} gradually disappearing and giving way to T_{CM}, most likely due to slow outgrowth of the T_{CM} [67,70]. Pathogen-specific memory T cells can persist in the tissues (such as the gut and lung) long-term [71,72], although these populations may be maintained by T_{CM} that migrate into tissues and form T_{EM} [73]. Transcription factors that control the T_{CM}/T_{EM} balance have also been identified. Activation of the PI3K/mTOR pathway downregulates CCR7 and CD62L expression via the transcription factor Klf2 [74•], suggesting metabolic control of the T_{CM}/T_{EM} balance. Furthermore, the transcription factors Id2, T-bet and Blimp-1 favour T_{EM} formation (RL Rutishauser and SMK, unpublished data) [40,57] whilst Bcl-6 promotes T_{CM} formation [75].

Conclusions

Whilst many cell intrinsic and extrinsic factors have been identified that shape $CD8^+$ T cell fate choices, it is still unclear how the large array of lineage-determining factors synergise and/or integrate to fine-tune cell fate choices. In particular, understanding how different cell fates are maintained (particularly in the absence of antigen) and determining the extent of fate plasticity remains a future challenge. To this end, understanding the epigenetic establishment and maintenance of the transcriptional signatures underlying different $CD8^+$ T cell fates represents an exciting area of future research.

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