Unrecognizable Memory Phenotype CD8+ T-cells

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Virtual memory T (TVM) cells are a recently described population of conventional CD8+ T cells that, in spite of their antigen inexperience, express markers of T cell activation. TVM cells exhibit rapid responsiveness to both antigen-specific and innate stimuli in youth but acquire intrinsic antigen-specific response defects in the elderly.

virtual memory T cells | Eomes | IL-15 | CD8+ T cells

1. Introduction

Traditionally, $CD8^+$ T cells have been considered to exist along a single spectrum; resting naïve $CD8^+$ T (T_N) cells, upon recognition of cognate antigen and subsequent activation, differentiate into effector T cells, which contract upon antigen clearance, leaving a conventional memory T (T_{MEM}) cell population. The T_{MEM} cell population is comprised largely of effector memory T cells (T_{EM}), found predominantly in tissues and primed for rapid effector function, and central memory T (T_{CM}) cells, found mainly in lymph nodes and responsible for self-renewal and supplying the pipeline of effector T cells [1]. T_{MEM} cells are quiescent but poised for activation, and present at a relatively high antigen-specific frequency. These features of $CD8^+$ T cell memory underpin their ability to respond rapidly after reencounter with the same antigen and are a hallmark of adaptive immunity. Recently, a novel population(s) of $CD8^+$ T cells has been identified, referred to variously as virtual memory T (T_{VM}) cells, memory phenotype (MP) T cells, antigen-inexperienced memory T (T_{AIM}) cells, and innate memory T (T_{IM}) cells, that exhibit many characteristics of T_{MEM} cells - including cell surface phenotype and rapid responsiveness to both antigen-specific and innate stimuli - despite having not previously encountered specific antigen. Herein, we generally refer to this population of antigen-naïve memory phenotype CD8+ T cells as T_{VM} cells. The interest in T_{VM} cells stems from their ability to exert robust and rapid effector functions never previously attributed to antigen-inexperienced T cells, their responsiveness to both antigen-specific and innate stimuli, their superior survival capacity and their intrinsic dysfunction in elderly mice and humans [2][3][4][5][6]. The following review highlights how T_{VM} cells have blurred the traditional boundaries between T_N cells and T_{MEM} cells and have driven the need for a re-evaluation of conventionally accepted T_{MEM} characteristics. In addition, we discuss recent advances in our understanding of T_{VM} cells, including their development as a distinct cell lineage and their biological relevance in protection from infection and cancers[7][8][9][10][11].

2. Conflation of Mouse T_{VM} cells with Conventional T_{MEM} cells

In mice, the phenotype of conventional T_{MEM} cell populations is well established [12][13] with all antigen-experienced memory T cells expressing the definitive activation marker, CD44, and differential expression of the lymph node homing receptor, CD62L, allowing distinction between T_{CM} and T_{EM} subsets [14]. However, the discovery of T_{VM} cells has revealed a substantial overlap in the cell surface phenotype of conventional T_{CM} cells and T_{VM} cells in mice, with both expressing high levels of CD44 and CD62L [15][16]. Consequently, T_{VM} cells have historically fallen into the conventional phenotypic definition of antigen-experienced $CD8^+$ T_{CM} population (Figure 1a). This can be easily overcome by the inclusion of CD49d, an integrin involved in cell trafficking, which is stably upregulated on T_{MEM} cells but, even with advanced age or certain infection models, remains low on T_{VM} cells. T_{VM} cells can also be identified by high level expression of IL-2Rβ/IL-15Rβ (CD122) compared to lower expression on T_{MEM} cells, reflecting T_{VM} cell sensitivity to IL-15 [17]. It has recently been demonstrated that conventionally defined T_{CM} cells are, in both young and aged mice, comprised predominantly (~80%) of T_{VM} cells. Even in mice recently infected with LCMV, which induces a robust $CD8^+$ T cell memory population, over 60% of $CD8^+$ $CD44^{hi}$ $CD62L^{hi}$ cells (i.e. conventionally defined T_{CM} cells) are T_{VM} cells [18]. Findings regarding the T_{CM} population may therefore be influenced by the inclusion of T_{VM} cells.

Figure 1. The Differentiation Continuum and Defining Phenotype of Steady-State $CD8^+$ T cells. $CD8^+$ T_{VM} cells are a semi-differentiated yet antigenically naïve T cell population, indicating the potential for antigen-independent T cell differentiation and representing a link between antigen naïve (yellow shaded) and antigen-experienced (red shaded) memory T cells. T_{VM} cells have historically been phenotypically included within (**a**) the T_{CM} cell population in mice, and (**b**) the T_{EMRA} cell population in humans. Lineage-defining markers (in bold), in conjunction with other additional markers, demarcate T_{VM} cells and reinforce their phenotypic and functional uniqueness.

Inaccurate attribution of characteristics as a consequence of the conflation of T_{VM} and T_{CM} cells is exemplified by our understanding of the reliance of $CD8^+$ T_{MEM} cells on IL-15 for survival. The current paradigm indicates that IL-15 is critical for T_{MEM} cell survival. However, recent studies have shown that in young and aged mice lacking IL-15 there is a complete loss of T_{VM} cells ($CD44^{hi}$ $CD49d^{lo}$) whilst T_{MEM} ($CD44^{hi}$ $CD49d^{hi}$) cells are relatively unaffected[19][20]. Similarly, in mice lacking CD122, the generation and maintenance of T_{MEM} cells remains relatively intact whilst T_{VM} cells fail to develop [17] These findings call into question other widely accepted characteristics of T_{CM} cells. Of particular interest is the dependence of T_{VM} and T_{CM} cells on tonic peptide + Major Histocompatibility Complex I molecule (MHCI)-TCR signalling for survival in the periphery. It has long been appreciated that circulating naïve $CD8^+$ T cells require low affinity self-pMHCI:TCR interactions in order to provide tonic signals for survival [21], contrasting memory cells whose survival is independent of MHCI but dependent on homeostatic cytokines such as IL-7 and IL-15 [22]. However, the requirements for survival of T_{VM} cells is contentious. Early adoptive transfer experiments demonstrated the survival of LCMV-specific memory phenotype (MP) cells in $\beta 2m^{-/-}$ hosts [23]. However, transferred MP cells in this experiment were defined only by high expression of CD44. Expanding on this finding, later adoptive transfer experiments demonstrated that MP cells expressing high levels of CD122 (characteristic of T_{VM} cells) were maintained in the periphery of MHC-$Ia^{-/-}$ mice, whilst MP cells expressing

low levels of CD122 failed to survive [24]. These CD122^{lo} MP cells expressed a cell surface phenotype reminiscent of recently activated effector CD8$^+$ T cells, including low expression of CD62L. Similarly, inspection of the endogenous population of peripheral CD8$^+$ T cells in MHC-I$^{-/-}$ hosts revealed the majority of these cells exhibited CD44^{hi} CD122^{hi} phenotype reminiscent of T_{VM} cells [25].-Thus, the extent to which conventional T_{CM} cells and T_{VM} cells rely on MHC-I for peripheral survival is yet to be definitively determined.

3. T_{VM} cells are Contained within the Human T_{EMRA} cell Population

While largely characterized in mice, a putative T_{VM} population has also been identified in humans, which displays both functional and phenotypic similarities to mouse T_{VM} cells [26]. These human T_{VM} cells display a differentiated phenotype typically associated with effector memory T cells re-expressing CD45RA (T_{EMRA}) (CD45RA$^+$CD27$^-$), express NK cell receptors (NKRs) such as KIRs and NKG2A, show high expression of Nur77 (indicative of high self-pMHCI affinity), and show high expression of the transcription factor Eomes, with rapid production of IFNγ upon innate-like stimulation. Furthermore, human T_{VM} cells accumulate with age and acquire defects in TCR-mediated proliferation. In addition to these parallels with mouse T_{VM} cells, human T_{VM} cells have been detected in human cord blood and thus their development appears to be independent of antigen exposure [26]. The limited study of human T_{VM} cells can be attributed to the lack of definitive surface markers that distinguish them from T_{EMRA} cells (CD8$^+$CD45RA$^+$CCR7$^-$). Currently, identification of human T_{VM} cells is based on the additional expression of NK cell markers, pan-KIR2D and KIR3DL, and/or NKG2A, which separates T_{VM} cells from the entire CD45RA$^+$ subset of CD8$^+$ T cells. Non-T_{VM} CD45RA$^+$ cells are further subdivided into T_N cells (CD27$^+$CD45RA$^+$CD95$^-$), T_{SCM} cells (CD27$^+$CD45RA$^+$CD95$^+$) [27] and T_{EMRA} cells. Owing to the overlap in surface marker expression between T_{EMRA} and T_{VM} cells, and the lack of routine inclusion of defining NKRs, T_{VM} cells are typically included within the T_{EMRA} population [28] (Figure 1b).

Despite their apparent similarities, there are key differences that distinguish T_{VM} cells from T_{EMRA} cells. Firstly, T_{VM} cells are considered to be antigen-inexperienced [29] whilst T_{EMRA} cells are antigen-experienced memory cells, as evidenced by the observation that they can comprise up to 39% of the CD8$^+$ T cells within a given epitope-specific population [30]. Secondly, T_{VM} cells have a higher proliferative capacity than T_{EMRA} cells which are non-proliferative in both young and aged individuals. Thirdly, although a direct comparison between human T_{VM} and T_{EMRA} cell metabolism has not been performed to date, our recent work in mouse models have shown that T_{VM} cells not only have the highest oxygen consumption rate (OCR) of all CD8$^+$ subsets in steady state but that it is further increased with infection and ageing. In addition, our study indicates that there is no difference in basal mitochondrial characteristics, such as mitochondrial mass and number of mitochondria per cell, between T_{VM} cells compared to other CD8$^+$ subsets. In contrast, T_{EMRA} cells have a lower basal OCR and extracellular acidification rate (ECAR), following overnight CD3 stimulation [31], as well as lower basal mitochondrial mass and fewer mitochondria per cell compared to conventional memory subsets [32].

The inclusion of NKRs to separate putative T_{VM} cells from T_{EMRA} cells in humans marks the beginning of the quest to better investigate this distinct cell population. It is clear that this putative T_{VM} population parallels many of the

functional characteristics observed in murine T_{VM} cells, emphasising the need to identify unique and definitive markers for future studies. Indeed, a recent single cell transcriptional analysis of human memory T cells has identified novel subsets of stem-like $CD8^+$ memory T cells, highlighting the heterogeneity that has confounded a complete understanding of memory phenotype T cells in humans.

4. Heterogeneity within the T_{VM} cell Compartment

Broadly, two populations of antigen-inexperienced MP cells have been described – T_{VM} cells and T_{IM} cells. Whilst T_{VM} cells and T_{IM} cells were originally distinguished from one another by the thymic expression of CD49d, their dependence on IL-15 vs IL-4, and their emergence in the periphery versus the thymus, the two populations are indistinguishable once in the periphery [33][34]. It is likely that these cells represent the same population, but their original identification in different mouse strains has resulted in the attribution of distinct characteristics. T_{IM} cells are highly abundant in BALB/c mice due to the ability of unconventional $PLZF^+$ NKT cells in this strain to produce large amounts of IL-4, facilitating T_{IM} differentiation [33][34][35] In contrast, T_{IM} cells are not readily detectable in C57Bl/6 mice, however, genetic alterations in these mice, such as knockout of tyrosine kinases ITK and RLK, increases the number of IL-4 producing $PLZF^+$ NKT cells and, in turn, increases the number of detectable T_{IM} cells within the thymus and periphery [36][37][38][39]. Moreover, while not readily detectable in a WT C57Bl/6 mouse thymus, recent evidence indicates T_{VM} cell differentiation is programmed during thymic development (see below) [40][41][42].

Although peripheral T_{VM} cells in mice are readily identified using cell surface markers such as CD49d, CD44 and CD122, whether or not this population represents a homogenous population of cells or an amalgamation of disparate cell subsets is unclear. Heterogeneity within the T_{VM} population has been suggested by a recent study which used tamoxifen-induced time stamping to analyse T_{VM} cells generated in the neonatal period (day 1) or later in life (day 28) [43]. T_{VM} cells generated early in life (day 1) exhibit a transcriptional profile more akin to a short-lived effector cell (SLEC), as indicated by expression of *tbx21*, *Ifng* and *gzma* genes, compared to those generated later in life (day 28). This heterogeneity translated to differences in functionality, with day 1 T_{VM} cells responding more rapidly to antigen and inflammatory cues linked with increases in effector molecules such as granzyme B and IFNγ. This exaggerated effector response also translated to a greater propensity to adopt a terminally differentiated ($KLRG1^{hi}$ $CD62L^{lo}$) phenotype 41 days post-infection.

Functional heterogeneity is further observed in a subset of T_{VM} cells that selectively express NK cell markers. NKR expression is a distinguishing feature of mouse T_{VM} cells [44], and a defining characteristic of human T_{VM} cells. Whilst NKR expression on memory $CD8^+$ T cells has conventionally been associated with senescence [45], in T_{VM} cells this subset appears to show heightened functionality, as evidenced by an increased ability to kill MHC-I deficient tumour cells following chemotherapy treatment in both humans and mice(Figure 2).

Figure 2. Virtual memory $CD8^+$ T cells (T_{VM}) participate in various immune responses to pathogens and tumors and may be involved in immune regulation. Boxes indicate the role and possible mechanism of action of T_{VM} cells in different disease or immune contexts.

Adding to evidence of functional heterogeneity within the T_{VM} population, a number of studies have indicated a regulatory role of a subset of $CD8^+CD44^{hi}CD122^{hi}$ cells. Early reports have suggested MP $CD8^+$ T cells function similar to that of CD4 T_{reg} cells via IL-10-induced suppression of effector function in activated CD4 and $CD8^+$ cells [46] (Figure 2). Later studies revealed that only the PD-1 negative MP subset displayed regulatory functions [47]. In addition, Akane and colleagues characterised these $CD8^+CD44^{hi}CD122^{hi}$ T_{reg} cells and determined they could be further defined from other $CD8^+$ MP cells via a lack of CD49d expression, suggesting they were in fact T_{VM} cells [48]. Taken together, these data suggest both phenotypic and functional heterogeneity within the T_{VM} population.

5. Heightened TCR Reactivity and Cytokine Sensitivity are Key T_{VM} cell Characteristics

TCR reactivity appears to be a key determinant in driving T_{VM} differentiation, phenotype and effector function. Firstly, T_{VM} cells have been shown to express heightened levels of CD5 in mice [49], and Nur77 in humans , which are surrogate markers for TCR signal strength [50][51] and thus are indicative of heightened TCR self-reactivity [52]. In addition, TCR repertoire analyses shows a TCR bias in $CD8^+$ MP cells [53][54], further suggesting the TCR dependence of T_{VM} differentiation. It is likely that this high self-peptide:MHCI reactivity during T_{VM} cell development drives the heightened T_{VM} cell cytokine sensitivity in the periphery, which can, at least in part, be attributed to Eomes expression. Eomes is a Tbox transcription factor which, in $CD8^+$ T cells, shows increased expression following activation [55]. In a study by Miller and colleagues, it was shown that Eomes expression could be upregulated during thymic maturation of $CD44^{hi}CD122^+$ cells, which was attributed to heightened TCR reactivity to self-ligands [53]. Eomes expression has also been shown to bind to the *il2rb* promoter leading to activation and a subsequent increase in CD122 expression [56]. Thus, the heightened self-peptide MHC reactivity of T_{VM} cells appears to upregulate Eomes expression, which in turn leads to increased CD122 expression, driving T_{VM} cell dependence on, and sensitivity to, IL-15. This is also supported by Gett and colleagues who showed that strong TCR engagement, and subsequent signalling, enhanced survival and responsiveness to IL-15, and other cytokines, through increased expression of cytokine receptors [57].

Eomes expression in T_{VM} cells has also been shown to be augmented by type I IFN signaling [58]. Indeed, IFNb signalling resulted in an Eomes-dependent increase of both peripheral T_{VM} cells and thymic T_{IM} cells, and T_{VM} cells were significantly diminished in $IFNAR^{-/-}$ mice [58]. Given the observation that tonic type I IFN signalling is received by SP thymocytes as a normal part of T cell development [59], it seems plausible that type I IFN signalling is essential both in the thymus for T_{VM} lineage differentiation at this SP stage [53], as well as in the periphery for the peripheral maintenance of Eomes expression.

Although a characteristic of memory cell subsets in general, T_{VM} cells are particularly sensitive to a range of homeostatic cytokines, such as IL-12, IL-18, IL-4, and IL-7 [60][61][62]. As mentioned, their particularly high sensitivity to IL-15 is likely to be due to increased expression of CD122 , which increases further with age, and leads to a downstream increase in STAT5 phosphorylation following stimulation with IL-15, compared to T_{MEM} cells [63][64][65]. Although the selective impact of cytokines on T_{VM} cells may correspond in part to changes in cytokine receptor expression, age-related changes in T_{VM} cell frequency and function may also be explained by an increase in the

levels of these cytokines with aging. For example, there is evidence for elevated IL-15, IL-6, IL-18 and TNF cytokine levels with advanced age, as part of the 'inflammaging' process [66][67][68][69]. Outside of IL-15, T_{VM} cells from both mice and humans can be directly activated by other cytokines. Previous *in vitro* studies of T_{VM} cells have shown that IFNγ production in these cells can be driven by IL-12 and IL-18 stimulation and result in an antigen-independent acquisition of cytotoxic capacity.

The role of cytokines in mediating the expansion and effector function of T_{VM} cells is further reinforced *in vivo* in the context of infection. Baez and colleagues demonstrated that mice infected with *Trypanosoma cruzi* showed enhanced expansion of $CD44^{hi}$ $CD8^{+}$ T cells, owing to increased levels of thymic IL-15 and IL-4, which ultimately promoted antigen-independent proliferation and subsequent protection from parasitemia by this population [70] (Figure 2). Furthermore, the ability of $CD44^{hi}NKG2D^{+}$ $CD8^{+}$ T cells to directly kill *Listeria monocytogenes*-infected target cells occurred independently of strong TCR signalling, but was instead NKG2D-dependent and promoted by direct cytokine exposure (IL-12, IL-15 and IL-18) [71] (Figure 2). This innate-like response was required for effective bacterial clearance during the acute stages of infection [71]. In the context of helminth infections, studies have shown that the robust IL-4 production following infection of B6 or BALB/c mice drives antigen-independent T_{VM} cell expansion, which in turn offered significant protection following subsequent viral or bacterial infections, via either innate or antigen-specific mechanisms [70].

Given the heightened cytokine sensitivity of T_{VM} cells, it will be of interest to determine whether changes in the cytokine milieu associated with infections over a life course, or that occur as a natural part of the aging process ('inflammaging'), are responsible for changes in T_{VM} cell number and function with age. In this way, it seems possible that the same cytokine responsiveness that may impart the rapid responses and semi-differentiated phenotype in T_{VM} cells from young mice and humans, might also be responsible for the acquisition of a senescent phenotype in advanced age.

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