Review

Hiding in Plain Sight: Virtually Unrecognizable Memory Phenotype CD8$^+$ T cells

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Abstract: Virtual memory T (T_{VM}) cells are a recently described population of conventional CD8$^+$ T cells that, in spite of their antigen inexperience, express markers of T cell activation. T_{VM} cells exhibit rapid responsiveness to both antigen-specific and innate stimuli in youth but acquire intrinsic antigen-specific response defects in the elderly. In this article, we review how the identification of T_{VM} cells necessitates a re-evaluation of accepted paradigms for conventional memory T (T_{MEM}) cells, the potential for heterogeneity within the T_{VM} population, and the defining characteristics of T_{VM} cells. Further, we highlight recent literature documenting the development of T_{VM} cells as a distinct CD8$^+$ T cell lineage as well their biological significance in the context of disease.

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 [2–6]. 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 [7–11].

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 [6,15,16]. 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 [3,6]. 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 [3]. Even in mice recently infected with LCMV, which induces a robust CD8$^+$ T cell memory population [3], over 60% of CD8$^+$ CD44^{hi} CD62L^{hi} cells (i.e. conventionally defined T_{CM} cells) are T_{VM} cells [3,18]. Findings regarding the T_{CM} population may therefore be influenced by the inclusion of T_{VM} cells.

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 [3,6]. 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 [3,19,20]. 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, 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 β2m$^{-/-}$ 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.
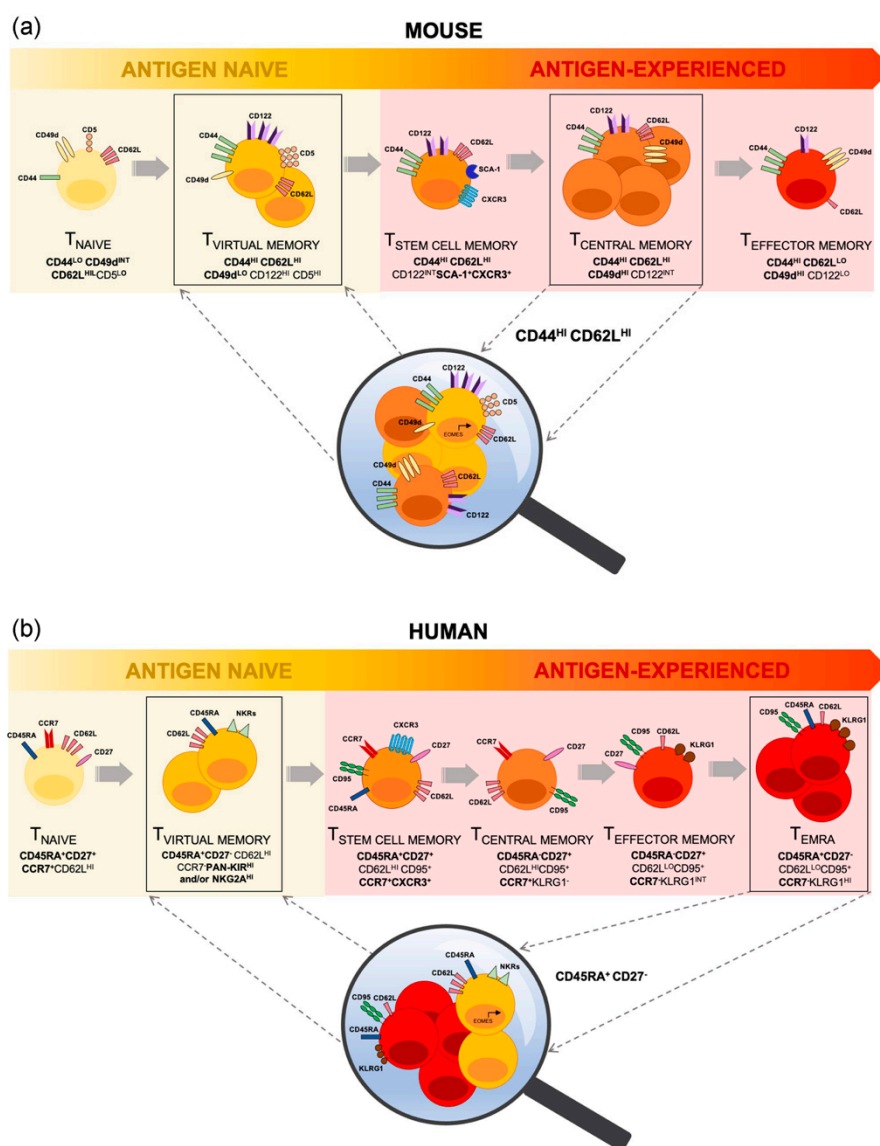
Figure 1. The Differentiation Continuum and Defining Phenotype of Steady-State CD8$^+$ T cells. CD8$^+$ T$_{VM}$ cells are a semi-differentiated yet antigenically naïve T cell population, indicating the potential for antigen-independent T cell differentiation and representing a link between antigen naïve (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.

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 [20,26]. 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) [20], and show high expression of the transcription factor Eomes, with rapid production of IFNγ upon innate-like stimulation [26]. Furthermore, human T$_{VM}$ cells accumulate with age and acquire defects in TCR-mediated proliferation [2,20]. 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 [5,29] 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 [2]. 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 [3]. 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 [3]. 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 [27], 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 [6,17,33,34]. 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 [33–35]. 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 [34,36–39]. 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) [40–42].

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 [20]. NKR expression is a distinguishing feature of mouse T_{VM} cells [6,20,44], 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 [9] (Figure 2).
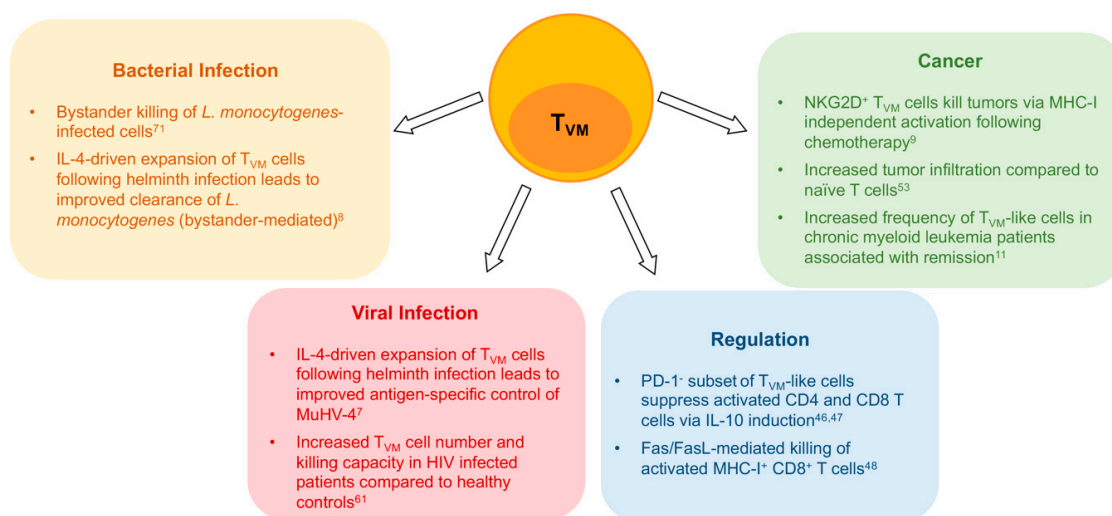
Figure 2. Virtual memory CD8$^+$ T cells (T_{VM}) participate in various immune responses to pathogens and tumors and may be involved in immune regulation. Boxes indicate the role and possible mechanism of action of T_{VM} cells in different disease or immune contexts.

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 [20], 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 [49,53,54], 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, IFNβ 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 [17,20,60–62]. As mentioned, their particularly high sensitivity to IL-15 is likely to be due to increased expression of CD122, which increases further with age [3], and leads to a downstream increase in STAT5 phosphorylation following stimulation with IL-15, compared to T$_{MEM}$ cells [2,5,60,63–65]. 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 [66–69]. 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 [6,20,60].

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 [7,8,70].

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.

6. NKR Expression as Markers of Functionality and Senescence on T$_{VM}$ Cells

It has been shown in both mice and humans that a subset of memory phenotype CD8$^+$ T cells express NK cell markers, with their frequencies increasing as a result of the ageing process [72–74] as well as during infection [75,76]. T$_{VM}$ cells have been reported to express NKRs [20] and appear

to demarcate a subset of functionally distinct cells within this population. Following TCR-mediated stimulation, human CD8^{+} T cells expressing NKRs, particular inhibitory NKRs such as NKG2A, KIR2DL and KIR3DL, show reduced effector cytokine production, compared to NKR^{−} CD8^{+} T cells, [77]. Despite this, KIR/NKG2A^{+} CD8^{+} T cells, in contrast to their KIR/NKG2A^{−} counterparts, were able to elicit strong TCR-independent cytotoxic responses via CD16 ligation and enhanced degranulation upon recognition of MHC class I-deficient target cells [26]. Thus, at a functional level, NKR-expressing CD8^{+} T cells appear to display reduced TCR^{−} mediated responses coincident with heightened innate responsiveness.

T cell expression of NKRs is broadly used as an indicator of cellular senescence. As reviewed by Michel and colleagues, the expression of NKRs on CD8^{+} T cells has been linked with classical properties of senescence, including increased expression of cell cycle arrest molecules, such as p53, as well as the markers KLRG1 and CD57, which have been shown to be expressed on terminally differentiated T cells which show poor TCR-dependent signalling activity [78]. Certainly, senescent cells, including senescent T_{VM} cells in aged mice, are characterised both by an upregulation of NKRs and enhanced longevity associated with high Bcl-2 expression [2]. Studies of both mouse and human T cells have shown that expression of inhibitory NKRs results in a decrease in activation-induced cell death in vitro [72,79–81]. Moreover, transgenic mice expressing the human KIR2DL3 and HLA-Cw3 ligand showed that ligation of inhibitory KIRs by MHC class I directly resulted in an in vivo accumulation of T_{VM}-like cells [72], directly demonstrating a linkage between NKR expression on T cells and enhanced survival, a key characteristic of senescent cells.

NKG2D is another important NKR that can be upregulated on both conventional T_{MEM} cells and T_{VM} cells after activation, and is typically associated with a senescent phenotype when expressed on CD8^{+} T cells [82]. However, despite being a marker of senescence and TCR-mediated dysfunction, the activity of NKG2D on these cells actually facilitates enhanced innate responsiveness. TCR-independent cytotoxicity has been observed following NKG2D engagement on memory phenotype CD8^{+} T cells, through measurement of increased granzyme and perforin production [26,83,84]. In addition, NKG2D has been shown to be expressed on senescent human CD8^{+} T cells and, through its interaction with the ITAM-containing DAP12 protein and Sestrin-2 (SESN2), was able to result in direct activation, cytokine production and cytotoxicity following ligation with its ligand, MICA, or through stimulation with anti-NKG2D antibody [45]. The SESN2-mediated augmentation of NKG2D expression and signalling, along with other NKRs, was concomitant with a downregulation of TCR-mediated signalling. Accordingly, inhibition of SESN2 resulted in a restoration of TCR-mediated signalling and a reduction in NKG2D-mediated, antigen-independent effector function. In mice, this reciprocal TCR-mediated and innate functionality of T cells is exemplified in a study by Anfossi and colleagues who showed that expression of inhibitory Ly49 molecules diminished TCR-mediated T cell activation without loss of sensitivity to IL-15-mediated activation [85]. These data provide a mechanistic explanation for the aforementioned observations of reduced TCR-mediated signalling in T cells expressing NKRs and indicate a reciprocal relationship between innate and adaptive functionality in CD8^{+} MP T cells.

This reciprocal functionality is relevant to the remodelling of function that is observed in both highly differentiated CD8^{+} T_{EMRA} cells in humans (that contain the T_{VM} population) and in T_{VM} cells from aged mice. Namely, the heightened expression of NKRs and sensitivity to cytokines such as IL-15 in advanced age, likely indicates a transition from classical TCR mediated functionality in young individuals, to a primary role in innate effector function, which has been proposed to be beneficial for tumour surveillance and the elimination of senescent cells in tissues [45]. These data highlight the importance of understanding the changing dynamic of T cell stimuli and effector functions over time, and the consideration that the observed 'senescence' of CD8^{+} T cells may simply indicate an advantageous functional adaptation in advanced age.

7. Transcriptional Regulation of T$_{VM}$ Development and Function

The formation of long-lived CD8$^+$ T cell memory after primary responses relies heavily on the binding of early transcription factors (TFs), such as RUNX3, TCF-1 and FOXO1, at pro-memory genes [86–88] facilitating chromatin remodelling. These early changes allow memory CD8$^+$ T cells to adopt a 'poised' chromatin conformation, allowing rapid recall responses via binding of TFs, such as T-bet, at effector gene loci [87]. Similarly, particular TFs have also been implicated in the development or differentiation of T$_{VM}$ cells. As mentioned earlier, the T-box TF Eomes is highly expressed by T$_{VM}$ cells in mice and humans and is critical for T$_{VM}$ formation [6,17]. Importantly these studies demonstrated that early Eomes upregulation in developing CD8$^+$ T cells in the thymus prior their adoption of a memory-like (CD44^{hi}CD122$^+$) phenotype is critical for their development [53]. Other groups have also shown that overexpression of Eomes alone was sufficient to drive antigen inexperienced cells towards a memory phenotype [89]. These studies not only solidify the importance of Eomes in T$_{VM}$ cell development, but also suggest that these cells represent a distinct lineage of CD8$^+$ T development (rather than an activation or differentiation stage) that is ratified in the periphery through IL-15 exposure [53]. This substantially changes the paradigm for T$_{VM}$ cell development, which previously considered these cells to arise largely as a consequence of homeostatic proliferation.

Eomes expression likely drives T$_{VM}$ differentiation via its promotion of CD122 expression by binding directly to the *il2rb* promoter [53,56,90]. In addition, it has been shown that Eomes is important for the programming of active enhancers in antigen-naïve MP cells, including T$_{VM}$ cells [89], via binding to RUNX3-bound enhancers, allowing for the chromatin remodelling protein BRG1 to bind and facilitate loop formation. RUNX3 is a critical transcription factor responsible for memory cell reprogramming following TCR stimulation via repression of gene transcription associated with terminal differentiation [88].

T$_{VM}$ development also appears to be controlled by negative transcriptional regulators, such as Bcl11b. Bcl11b is first expressed at the DN2 stage of thymocyte development and its maintenance is required to suppress innate gene expression, including some NKRs and Runx3 [41]. Despite its critical role in regulating positive and negative selection within the thymus [91,92], mice lacking a functional form of Bcll1b accumulated thymocytes and peripheral CD8$^+$ T cells with a memory phenotype (CD44^{hi}CD122^{hi}) that remain functionally active [40–42]. These MP cells upregulated transcripts of NKRs as well as increased Eomes expression contributing to their high expression of CD122 [40]. The increase of these cells was found to be due to a cell intrinsic action of Bcl11b and not secondary to elevated thymic IL-4 availability as observed in other genetically modified mouse models [29,34,36–39,44]. Thus, T$_{VM}$ cell differentiation appears to be tightly controlled through the coordinated action of both positive and negative transcriptional regulation acting in the thymus during T cell development.

8. Epigenetic Regulation of T$_{VM}$ Cells and Age-Related T Cell Dysfunction

The epigenome has been shown to be a critical determinant of CD8$^+$ T cell development as well as the ability of CD8$^+$ T cells to mount a robust antigen-specific effector response to infections such as Influenza A virus [93–97]. However, little is currently known about the role of epigenetics in the development of T$_{VM}$ CD8$^+$ T cells, particularly the development and changing function of T$_{VM}$ cells with age [3,6,18,44]. In the previously mentioned study by Smith and colleagues, it was demonstrated that T$_{VM}$ cell developmental timing determined responsiveness to effector cues and also impacted T$_{VM}$ cell epigenetic identity [43]. The heightened semi-differentiated phenotype seen in day 1 compared to day 28 T$_{VM}$ cells was associated with an increase in chromatin accessibility at gene loci associated with effector function (e.g., *gzma*, *Ifng*, *tbx21*) facilitating rapid transcription. These epigenetic changes resulted in increases in granzyme B and IFNγ production as well as increases in early response to bacterial challenge [43]. Thus, day 1 T$_{VM}$ cells displayed an epigenome pre-programmed towards a SLEC response rather than an MPEC response [98]. These data indicate

that T_{VM} functionality, and dysfunction, is likely controlled via underlying epigenetic mechanisms reflected by chromatin accessibility.

Of particular interest is whether the key functional changes associated with T_{VM} cells in aged mice (>18 months), namely the development of cellular senescence and the retention of cytokine responsiveness, are also reflected by epigenetic changes. Further, it is unclear whether the epigenome of aged T_{VM} cells reflects that which is typically observed in conventional memory T cells that have undergone proliferative senescence [99,100]. Broadly, ageing cells tend to exhibit many different epigenetic changes, including the loss of histone proteins, imbalances in post translational modifications, site-specific gains in heterochromatin and aberrant changes in chromatin accessibility [101]. All of these changes result in chromatin instability, transcriptional noise and impaired DNA damage repair all of which contribute to the accumulation of senescent cells as well as the pathology observed with age [102,103]. In an effort to understand the role of epigenetics in the regulation of T-cell specific age-related changes, studies have used ATAC-seq to examined the changes in CD8$^+$ T cell chromatin accessibility [104,105]. These experiments demonstrated concentrated changes in chromatin accessibility within aged CD8$^+$ T cells, specifically those possessing a memory phenotype. Moskowitz et al demonstrated that CD8$^+$ T_N and T_{CM} cells adopted a chromatin organisation more akin to differentiated CD8$^+$ T cells via their increased accessibility of the bZIP family of transcription proteins, such as BATF, which is critical for effector differentiation [106].

Another significant observation made by these studies was a broad preferential loss in accessibility at promoters rather than enhancers[4], with affected promoters enriched for NRF1 binding [105] as well as the observed closing at the *il7r* gene promoter [104]. Sites of chromatin closing were also found to be enriched for genes associated with TCR signalling, emphasising the profound effect ageing has on normal T cell function. These studies have provided powerful insight into the molecular mechanisms that underpin human CD8$^+$ T cell ageing, but detailed studies on the epigenetics of dysfunctional CD8$^+$ T cell subsets, such as T_{VM} cells, are lacking.

Although clearly important in the differentiation of T_N cells to effector T cells and then in the establishment of antigen-specific memory, the role of histone post-translational modifications (PTMs) on T_{VM} cell development and function is poorly studied. However, one key study has revealed an important role for the H3K79 methyltransferase, DOT1L, in T_{VM} cell development [107]. In this study, it was demonstrated that, in the absence of DOT1L from T cells, almost all CD8$^+$ T cells exhibited a T_{VM} cell phenotype. Due to the almost complete absence of circulating T_N cells in these mice, the authors suggested a role for H3K79me in sustaining the naive state in CD8$^+$ T cells, and that deletion of DOT1L may drive cells down a semi-differentiated state independently of antigen exposure [107]. These T_{VM}-like cells also appeared during thymic development, suggesting that, similar to Bcl11b, Dot1L is required to suppress development of T cells down a T_{VM} lineage. Further, detailed studies investigating histone PTMs, and epigenetic changes more generally, in the development and maintenance of T_{VM} cells is essential for our understanding of the biology of this population and, by extension and comparison, other CD8$^+$ T cell populations.

9. Concluding Remarks

As new information emerges it is becoming clearer that memory phenotype T_{VM} cells have a unique function within the immune system, that may evolve quite dramatically over a life course. The identification of this distinct CD8$^+$ T cell lineage necessitates a re-evaluation of many of the current paradigms associated with conventional antigen-experienced memory CD8$^+$ T cells. Further study should ascertain the heterogeneity of phenotype and function within the T_{VM} population, which should in turn allow for a more precise definition of this population, especially in the human context. Finally, the recent delineation of T_{VM} cells as a distinct developmental pathway, and the identification of several transcriptional and epigenetic controllers of this fate, has highlighted the active mechanisms in place to maintain the naïve CD8$^+$ T cell state and prevent excessive T cell activation/innate functionality. Thus, further investigation of T_{VM} populations, while inherently important for our understanding

of T_{VM} cell biology, should also serve to advance our understanding of molecular controls that are essential for maintaining adaptive functionality and naïve state of conventional CD8$^+$ T cells.

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