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REVIEW

Quick to remember, slow to forget: rapid recall responses of memory CD8$^+$ T cells

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The functional roles of memory B and T lymphocytes underlie the phenomenal success of prophylactic vaccinations, which have decreased morbidities and mortalities from infectious diseases globally over the last 50 years. However, it is becoming increasingly appreciated that memory cells are also capable of mediating the pathology associated with autoimmune disorders and transplant rejection, and may pose a significant barrier to future clinical advancement in immunoregulation. Therefore, understanding the unique properties of memory lymphocytes (as compared to their naive precursors) is a major area of investigation. Here, we focus on one of those singular properties of memory T cells (T_M)—rapid recall. As will be discussed in more detail, rapid recall refers to the ability of quiescent T_M cells to efficiently and robustly express 'effector functions' following stimulation. Studies that have advanced our understanding of T_M cells' rapid recall using CD4$^+$ T cells have been expertly reviewed elsewhere [1], so we will focus primarily on studies of CD8$^+$ T cells. We will first review the different ways that CD8$^+$ T_M cells can be generated, followed by discussing how this influences their functional properties in the settings of immune protection and pathology. Then, rapid recall ability will be discussed, with emphasis placed on what is currently known about the mechanisms that underlie this unique property of T_M cells.

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The generation of CD8$^+$ T_M cells

Memory T cells are derived from naïve precursors (T_N cells); however, research over the past 20 years has revealed a surprising number of contexts in which this can occur [2-4]. They all involve a signal received through the T-cell receptor (TCR), via its interactions with foreign or self antigens (Ag) and can take place in an inflammatory setting (such as infection) or in the presence of elevated levels of pro-survival and proliferation cytokines during lymphopenia.

Acute pathogenic infections

The process of CD8$^+$ T_M-cell differentiation in the context of acute pathogenic infection has been characterized in the most detail [5, 6]. Rare T_N cells, specific for pathogen-derived peptides, encounter Ag-presenting cells (APC) in lymph nodes draining infected tissues, where T_N cells receive a combination of signals from the TCR, co-stimulatory molecules and cytokine receptors [5, 7]. In this context, T_N-cell activation results in massive proliferation (clonal expansion phase), where the number of CD8$^+$ T cells specific for a given viral peptide can increase by as much as 50 000-fold over 7-8 days [8-11]. During this time, differentiation to effector T cells (T_E) occurs, with CD8$^+$ T cells developing the ability to migrate to peripheral tissues, abundantly secrete pro-inflammatory cytokines (such as TNFα and IFNγ) and lyse infected target cells [6]. Following pathogen clearance, 90-95% of the T_E cells die (contraction phase), leaving behind a population of pathogen-specific CD8$^+$ T_M cells that is maintained at a remarkably stable level for the lifetime of the host [11].

Following an acute infection, the generation of a CD8$^+$ T_M-cell population effectively increases the number of pathogen-specific CD8$^+$ T cells, giving the T_M-cell population a numerical advantage over their T_N-cell

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counterparts in controlling subsequent challenges by the same pathogen. Thus, this quantitative increase in precursor frequency is one of the distinguishing features of immunological memory [12, 13]; and long-term maintenance of CD8$^+$ T_M-cell populations is achieved via slow, Ag-independent turnover of individual T_M cells [14-17]. However, this 'homeostatic proliferation' is not the only property that distinguishes T_M cells from their T_N-cell precursors. Specifically, there are subsets of T_M cells that can migrate to peripheral tissues, enhancing surveillance of pathogen entry sites [18-21]. Furthermore, after stimulation, CD8$^+$ T_M cells may more rapidly enter and progress through the cell cycle than T_N cells [22-24]. Lastly, another distinctive feature of CD8$^+$ T_M cells is their ability to robustly produce cytokines and kill infected target cells within hours following stimulation. This contrasts with their T_N-cell precursors, which require replication and differentiation over several days to achieve the same functional capacity [22, 25, 26]. Together, heightened precursor frequency, expanded anatomical distribution, enhanced proliferative capacity and rapid recall ability comprise the hallmark attributes of protective CD8$^+$ T_M cells.

Lymphopenia-induced proliferation

In addition to infection-induced proliferation, CD8$^+$ T_N cells are also stimulated to divide under conditions of lymphopenia, such as that found in neonates or after lymphoablative therapies for cancers. Surprisingly, this does not result in 'homeostatic proliferation,' which would renew the T_N-cell population. Rather, under lymphopenic conditions, CD8$^+$ T_N cells can proliferate and differentiate into cells that possess the phenotypic and functional properties, as well as a gene expression profile, similar to that of T_M cells generated after pathogenic infections [4, 27, 28]. This phenomenon is termed 'lymphopenia-induced proliferation' (LIP) and it has several implications for our understanding of the contribution of CD8$^+$ T_M cells to immune protection and pathology.

Since the first demonstrations that LIP can change the surface marker profile of CD8$^+$ T_N cells into one that resembles that of T_M cells, several properties of these cells have been defined [4]. Importantly, like T_M cells formed after acute pathogenic infections, several groups have shown that CD8$^+$ T_M cells generated via LIP have cytotoxic ability, can efficiently upregulate production of effector cytokines and chemokines following stimulation and are capable of robust proliferation after Ag challenge *in vivo* [25, 29, 30]. Moreover, these cells are dependent on the presence of helper CD4$^+$ T cells (CD4$^+$ T_H) for normal differentiation, a requirement shared with CD8$^+$ T_M cells generated by acute infections [25, 31, 32]. It should be noted that CD8$^+$ T_N cells undergoing LIP do not appear to pass through a classical 'effector' stage of differentiation, characterized by the upregulation of the activation markers CD69 and CD25, nor do they downregulate the adhesion molecule CD62L, a process which facilitates homing to tissues by excluding these cells from lymph nodes [4, 28, 30, 33]. In fact, an important distinction between T_M cells generated via LIP and those generated by infection may be the ability of the latter to migrate to peripheral tissues. It is now well-known that T_M cells generated after acute infections are heterogeneous, even among a population with the same TCR specificity [19, 20]. Effector memory (T_{EM}) cells are characterized by low levels of the homing molecules CD62L and CCR7, excluding them from lymph nodes and facilitating their migration to peripheral tissues. Conversely, central memory (T_{CM}) cells express high levels of CD62L and CCR7, facilitating their accumulation in lymph nodes [34]. Whether this heterogeneity also exists within T_M cells generated via LIP is not yet clear. Nonetheless, the functional properties of CD8$^+$ T_M cells generated via LIP can bear a striking resemblance to those of T_M cells formed after acute pathogenic infections [4].

In summary, CD8$^+$ T_M cells generated after acute infections by intracellular pathogens have been called 'true' memory cells [25] and their importance to the efficacy of natural anamnestic responses, as well as those amplified by vaccination, is clear. However, over the past decade, it has been convincingly demonstrated that CD8$^+$ T_M cells can be derived from T_N cells that undergo proliferation and differentiation in a lymphopenic environment. Given that both types of CD8$^+$ T_M cells have the capacity to exert the effector functions that mediate protection and pathology, each will be discussed in these two contexts in the next section, with emphasis being placed on the distinctive rapid recall ability of CD8$^+$ T_M cells.

CD8$^+$ T_M cells' rapid recall in infection and disease

Together, the unique attributes of CD8$^+$ T_M cells – found in their frequencies, migration patterns, longevity and functional capacity – have led to a summation of CD8$^+$ T_M cells as both 'quantitatively and qualitatively' superior to their T_N precursors. Given the quantitative differences with T_N-cell populations, support for the importance of T_M cell qualitative enhancements has come largely from studies where equal numbers of T_N and T_M cells, specific for the same epitope, have been compared. Technically, this has been difficult to achieve, because of the very small number of CD8$^+$ T_N cells possessing the same TCR specificity in adult mice (estimated to be ~80-1 200 for any specific MHC I-restricted epitope [24,

35]). However, such experiments have been done with TCR transgenic (Tg) CD8$^+$ T cells, which are engineered to express TCRs that recognize epitopes from model Ags, such as ovalbumin, or model pathogens, such as lymphocytic choriomeningitis virus (LCMV) [36]. The absence of cognate Ag expression in TCR Tg mice results in a mostly naïve, monoclonal CD8$^+$ T-cell population (particularly when re-arrangement of non-Tg TCRs is suppressed by additional crossing onto RAG-deficient backgrounds). Adoptive transfer of Tg CD8$^+$ T_N cells to naïve host mice, followed by stimulation of the donor cells with cognate Ag, can generate a TCR Tg CD8$^+$ T_M-cell population. These T_M and T_N cells can then be purified and their properties compared on a 'per cell basis,' either *in vivo* or *in vitro*.

When equal numbers of CD8$^+$ TCR Tg T_N and T_M cells are adoptively transferred to separate naïve hosts, the T_M cells mediate superior protection following infectious challenge and they are uniquely able to clear chronic viral infections [37-39]. These T_M cells can be generated by adoptive transfer of CD8$^+$ TCR Tg T_N cells to naïve hosts, followed by infection with pathogens expressing the cognate epitope recognized by the Tg TCR. In addition, Jameson and colleagues demonstrated the ability of TCR Tg CD8$^+$ T_M cells generated via LIP to control pathogen replication more efficiently than their T_N precursors, indicating that pathogenic infection is not required to generate a population of highly functional, protective CD8$^+$ T_M cells [25, 40].

The enhanced protective capacity of T_M cells initially demonstrated in infection models predicted that CD8$^+$ T_M cells may be especially damaging in settings of transplant rejection and autoimmunity. Indeed, it was recently reported that CD8$^+$ T_M cells are superior to their T_N counterparts in their ability to reject an allograft. Wood and colleagues [41] utilized TCR Tg BM3 CD8$^+$ T cells, with a TCR specific for the MHC Class I allo-Ag H-2K^b. Naïve BM3 cells were adoptively transferred to RAG$^{-/-}$ hosts, which were then given an H-2K^b skin allograft, resulting in activation and conversion of the BM3 donor cells to a memory phenotype. The ability of BM3 T_N or T_M cells to reject an allograft was then compared by transfer of an equal number of T_N or T_M cells to RAG$^{-/-}$ hosts, which also received an H-2K^b skin allograft. The T_M cells mediated graft rejection more rapidly than their T_N counterparts (17 days versus 27 days mean graft survival time). This correlated with the ability of the T_M cells to efficiently produce IFNγ after stimulation with allo-Ag *in vitro* [41]. Notably, the absence of other lymphocytes in the RAG$^{-/-}$ recipients demonstrated the sufficiency of CD8$^+$ T cells for graft rejection, as RAG$^{-/-}$ recipients that did not receive CD8$^+$ T cells were 100% tolerant.

The enhanced pathological potential of CD8$^+$ T_M has also been observed in a mouse model of Type 1 diabetes. Hernandez and colleagues [42] utilized TCR Tg CD8$^+$ T

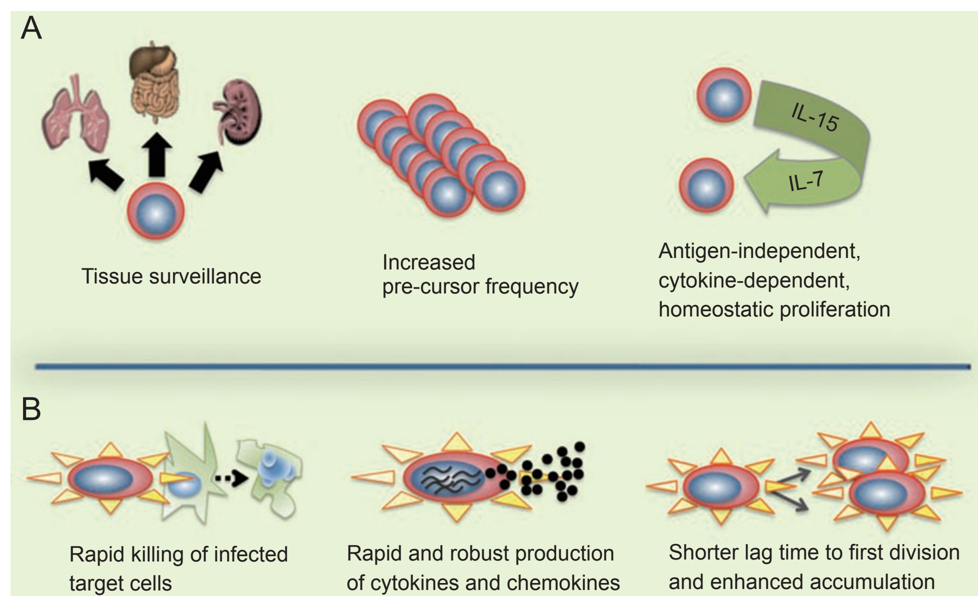

Figure 1 Unique properties of CD8$^+$ T_M cells. **(A)** At rest, as compared to their T_N counterparts, T_M cells are present at a higher precursor frequency, can migrate into non-lymphoid tissues and undergo antigen-independent turnover, which supports their long-term maintenance. **(B)** Following stimulation, as compared to their T_N precursors, CD8$^+$ T_M cells more efficiently increase cytokine and chemokine production, acquire cytotoxic ability and may also commence cell division earlier.

cells specific for a peptide from influenza virus. These cells were transferred into mice that expressed the viral peptide as a neo-self Ag exclusively in pancreatic beta cells, and that had also been rendered lymphopenic via irradiation. The TCR Tg cells underwent LIP, acquired a memory phenotype and were able to produce cytokines efficiently after stimulation with the specific peptide. Moreover, 100% of the recipient mice developed diabetes, while the same mice that had not been irradiated (and where differentiation of CD8$^+$ T_N to T_M cells did not occur) were healthy.

Here, a distinction can be made between the unique properties of 'resting' T_M cells (manifested prior to secondary infections and stimulations) and those of stimulated T_M cells (Figure 1). Specifically, in the absence of stimulation, T_M cells are distinct from T_N cells in their ability to migrate to peripheral, non-lymphoid tissues [20, 34]. Thus, while rapid recall likely contributed to the superior T_M cell-mediated protection and pathology in the studies described above, these adoptive transfer experiments cannot exclude the possibility that T_M cells only appeared to respond more quickly because they encountered Ag in peripheral tissues before their T_N counterparts. However, many experiments have demonstrated the enhanced functional capacity of T_M cells stimulated *in vitro*, where migration is not an issue and where the conditions of stimulation were more stringently controlled (such as APC number and peptide density). Following *in vitro* stimulation, T_M cells display enhanced proliferation and more efficient cytokine production than their T_N precursors (discussed below). Therefore, for the remainder of this review, we will refer to these stimulation-induced properties as the enhanced 'functional capacity' of CD8$^+$ T_M cells that form the essence of their rapid recall ability.

Phenotypes associated with CD8$^+$ T_M cells' rapid recall ability

Proliferation

A hallmark feature of both CD8$^+$ T_N and T_M cells is their ability for exponential proliferation following stimulation. However, whether T_M cells have an enhanced proliferative capacity (for example, begin dividing sooner after stimulation and/or display more rapid accumulation of progeny cells), is still controversial. Initially, Rocha and colleagues [22] reported that, following stimulation, CD8$^+$ T_M cells enter the cell cycle earlier than T_N cells and progress through it more rapidly. Using TCR Tg CD8$^+$ T_N or T_M cells (specific for a male HY Ag) adoptively transferred into separate female hosts immunized with male cells, they found that all T_M cells detected at 24 h after transfer had increased in size, indicative of the growth phase prior to cell division; whereas very few T_N cells were blasting at the same time point. Furthermore, mathematical modeling of their CFSE dilution profiles revealed that T_M cells had a shorter lag time to their first division (by 15 h) and progressed slightly faster through subsequent cell cycles than their T_N-cell counterparts [22]. These results have been criticized due to some particular properties of HY-specific TCR Tg cells that may not be representative of naturally occurring polyclonal CD8$^+$ T_N cells, including the fact that they do not undergo LIP [22, 28]. In addition, since the HY-specific T_M cells were generated in a non-infectious context, they may have been deprived of the abundant amounts of pro-inflammatory cytokines known to regulate T_M-cell differentiation during pathogenic infections [5, 43, 44]. Thus, their properties may not accurately represent pathogen-specific CD8$^+$ T_M cells. However, Ahmed and colleagues performed similar experiments using the LCMV infection model. They transferred equal numbers of LCMV-specific TCR Tg CD8$^+$ T_N and T_M cells into the same host, which was then acutely infected with LCMV. They found greater accumulation of progeny cells derived from the T_M-cell population at multiple time points post-infection [45]. Thus, these results using an infection model are consistent with the initial findings described above.

Differences in proliferative ability between CD8$^+$ T_N and T_M cells have recently been called into question by Carbone and colleagues. They compared TCR Tg CD8$^+$ T_N and T_M cells specific for a peptide from a glycoprotein of herpes simplex virus (HSV). To generate T_M cells, Tg T_N cells were adoptively transferred into naïve host mice, followed by infection of hosts with HSV [46]. The CFSE dilution profiles of these T_M and T_N cells were compared following stimulation with HSV peptide *in vitro* over several days. Cells in neither population had divided after 24 h, while the same percentage of T_N and T_M cells had divided at later time points and appeared to have undergone approximately the same number of divisions [46]. When these T_N and T_M cells were adoptively transferred into separate recipients challenged with HSV, the progeny of the T_N and T_M cells accumulated at the same rate in the spleen and trafficked equally well to the site of infection. Thus, at least in this model of localized HSV infection, there appeared to be no difference between CD8$^+$ T_N and T_M cells in their ability to proliferate in response to stimulation *in vitro* or accumulate in response to infection *in vivo*.

In summary, work in murine models has shown that when the number of precursor cells is controlled for, CD8$^+$ T_M cells can accumulate at the same rate or faster than T_N cells following stimulation, depending on the experimental system. These experiments have relied

exclusively on the use of TCR Tg cells, whose characteristics may not represent endogenous T_N and T_M-cell populations [5]. The recent advent of methodology that allows the purification of rare T_N cells should allow comparisons of non-Tg, polyclonal $CD8^+$ T_N and T_M cells. Using this method, Kedl and colleagues have compared naturally occurring $CD8^+$ T_N and T_M cells specific for the same viral epitope, and reported that a higher frequency of T_M than T_N cells had proliferated after 3 days of *in vitro* stimulation [24]. Lastly, $CD8^+$ T_M cells accumulated more rapidly *in vivo* than T_N cells following infection with either LCMV or vaccinia virus [45], but not with HSV [46], suggesting that the enhanced proliferative capacity of T_M cells may be particularly sensitive to the distinct inflammatory environments presented by different pathogens.

Cytokine production and cytotoxicity

Given the relatively rare frequency of pathogen-specific T_M cells and the rapid replication that microbes are capable of, it may be expected that the effector functions of $CD8^+$ T_M cells would not be limited to target cell lysis and might include the production of chemokines that can recruit innate immune cells, like macrophages and neutrophils, and cytokines that can activate them. In fact, an important part of $CD8^+$ T_M cells' rapid recall ability is their efficient upregulation of cytokine and chemokine production following stimulation. This phenotype is well established in infection and immunization models, and so we will briefly review this work before highlighting some more recent demonstrations of T_M cells' rapid recall in models of autoimmunity and transplantation.

As discussed, the use of monoclonal, TCR Tg cells has allowed comparisons of the properties of T_N and T_M cells on a per cell basis, controlling for potential differences in the number of cells in each population and in the affinity of TCRs that could be present in polyclonal populations specific for the same epitope. Furthermore, *in vitro* stimulation with specific peptide controls for potential differences in *in vivo* Ag accessibility. Using the HY TCR Tg system, Rocha and colleagues found that after 7 h of *in vitro* stimulation with peptide-pulsed splenocytes, ~70% of $CD8^+$ T_M cells expressed two or more cytokine mRNAs (IFNγ, IL-2 and/or Perforin), while double cytokine-producing T_N cells were not detected [22]. Similarly, $CD8^+$ T_M cells generated by LIP were also capable of efficient recall of cytokine production, as TCR Tg OT-I T_M cells (generated by transfer of OT-I T_N cells into partially irradiated congenic hosts) produced IFNγ, IL-2 and TNFα more rapidly than naïve (OT-I) cells after *in vitro* stimulation with specific peptide [25].

Recent studies have correlated the enhanced functionality of T_M cells with their potential pathological roles. Jones and colleagues used a model of alloreactivity with TCR Tg $CD8^+$ T cells specific for the MHC Class I allo-Ag H-$2K^b$ (BM3 cells). They adoptively transferred purified naïve ($CD44^{lo}$) BM3 $CD8^+$ T cells into congenic $RAG^{-/-}$ hosts, which received an allogeneic H-$2K^b$ skin graft. The grafts were rejected in a manner that generated a long-lived population of BM3 T_M cells [41]. Also, following overnight incubation with allogeneic H-$2K^b$-expressing stimulator cells, the BM3 T_M cells secreted more IFNγ than their T_N-cell precursors, demonstrating the efficient recall ability of $CD8^+$ T_M cells in a transplantation model [41]. Furthermore, cytokine production from T_M cells may contribute to pathology in the autoimmune disease rheumatoid arthritis (RA). Recently, it was shown that RA patients contain a population of peripheral blood T_{EM} $CD4^+$ T cells ($CD45RO^+CD45RA^-CCR7^-$) that produces IFNγ rapidly after stimulation with a set of cytokines present in inflamed joints, namely, IL-12, IL-15 and IL-18 [47]. Importantly, prior to stimulation, the same T_M cells expressed high levels of these cytokine receptors, suggesting that chronic, cytokine-mediated activation of T_M cells can in turn result in their production of additional pro-inflammatory cytokines, sustaining cycles of immune activation in autoimmune disease [47].

Lastly, the ability of stimulated $CD8^+$ T_M cells to rapidly lyse infected target cells was not initially appreciated based on *in vitro* assays [48], but more recent data indicate that T_M cells can kill extremely rapidly and efficiently *in vivo* [26, 49]. Using a novel *in vivo* cytotoxicity assay, Barber *et al*. showed that fluorescently-labeled target cells pulsed with LCMV-derived peptides were rapidly eliminated when transferred into LCMV-immune, but not naïve, mice. Specifically, 10-20% of peptide-pulsed target cells were eliminated by 1 h post-injection and 90% of target cells were eliminated within 4 h [26].

Remarkably, $CD8^+$ T_M cells are able to rapidly activate multiple effector cell functions within hours following stimulation, a feature that distinguishes them from their T_N-cell precursors. Unfortunately, current studies of recall responses have focused on a few known effector molecules, but it is likely that many factors are rapidly induced in T_M cells following stimulation that contribute to their enhanced functionality. This complicates a rigorous testing of the role that T_M cells' rapid recall ability has in the outcomes of immune responses. The test is further hampered by a lack of basic knowledge about the specific signaling pathways and crucial regulators involved, thus precluding their functional inhibition. However, the distinct mechanisms used by T_M cells to execute rapid recall responses are starting to be elucidated and will be the subject of the remainder of this review.

Mechanisms underlying CD8$^+$ T$_M$ cells' rapid recall ability

Although the rapid recall ability of CD8$^+$ T$_M$ cells is well-established, the mechanisms underlying this ability are only beginning to be discovered. It is important to consider that the proliferation, secretion of effector cytokines and manifestation of cytolytic ability by CD8$^+$ T cells all require the transmission of signal(s) received via the TCR and associated co-stimulatory molecules and/or cytokine receptors. Thus, there are many levels at which CD8$^+$ T-cell function can be regulated that could potentially differ between T$_N$ and T$_M$ cells. These include the 'relay' of TCR and cytokine receptor-mediated signals (via intracellular signaling cascades) and the 'execution' of the signals (via changes in protein activity and/or expression). A general theme that has been proposed to underlie rapid recall ability is that, even when at rest, T$_M$ cells exist in a 'ready-to-respond' state. There is now evidence to support this hypothesis at multiple levels of regulation.

TCR-proximal signaling events

Given the complex, polyclonal T-cell responses to pathogen-derived Ags, together with the exponential expansion and profound contraction of CD8$^+$ T cells during infection, it was initially proposed that CD8$^+$ T-cell clones with high affinity for foreign-peptide-MHC complexes were selectively expanded after infection and retained in the T$_M$-cell pool, thus explaining the efficacy of T$_M$-cell recall responses. However, in an elegant study designed to ask whether or not such 'affinity maturation' is necessary for the generation of a highly functional, pathogen-specific T$_M$-cell pool, Slifka *et al*. [50] found that CD8$^+$ TCR Tg cells, specific for an LCMV-derived peptide, developed the ability to respond to lower concentrations of peptide during the primary response to LCMV infection. Moreover, this heightened sensitivity (termed 'functional avidity maturation') was maintained at a population level long after the infection was cleared. The use of monoclonal, TCR Tg cells (on a RAG$^{-/-}$ background) eliminated the possibility that higher affinity TCR clones were selectively retained in the T$_M$-cell pool, and so the authors postulated that T$_M$ cells' heightened responsiveness involved an enhanced capacity for TCR-mediated signal transduction. Though this hypothesis was not directly tested, they did show that, on average, individual virus-specific effector CD8$^+$ T cells (at day 8 post-infection) contained a higher level of the TCR-associated tyrosine kinase Lck than their T$_N$-cell precursors, and that this higher level was maintained in resting T$_M$ cells [50]. These experiments first identified a positive correlation between levels of TCR-associated signaling components and CD8$^+$ T$_M$-cell cytokine production, a correlation that has been confirmed and extended by others [51]. However, precisely how higher resting Lck levels contribute to CD8$^+$ T$_M$ cells' enhanced functional capacity after stimulation remains to be determined.

Additional evidence suggested that signals received via the TCR may be relayed differently by CD8$^+$ T$_N$ and T$_M$ cells, and possibly in a manner that is more efficient in T$_M$ cells. It does not appear that there are differences in the levels of surface TCR or associated CD3 complex components between T$_N$ and T$_M$ cells [51]. However, Kersh *et al*. [51] found that, long after an acute viral infection had been cleared, virus-specific CD8$^+$ T$_M$ cells contained elevated resting levels of phosphorylated LAT, which is the active form of a TCR-proximal scaffolding molecule. This phenotype positively correlated with T$_M$ cells' more efficient accumulation of phosphorylated signaling molecules downstream of LAT (such as ERK 1/2, p38 and JNK2), as compared to T$_N$ cells, 15 min after *in vivo* injection of specific peptide. Together, these results suggested that pre-assembly of the TCR signal transduction cascade may facilitate more rapid signaling in CD8$^+$ T$_M$ cells.

Furthermore, using a Tg mouse model, where expression of the TCR proximal kinase Lck was controlled by a tetracycline-responsive promoter, Zamoyska and colleagues [52] found that CD8$^+$ T$_M$ were fully able to produce effector cytokines in response to specific peptide stimulation without Lck, demonstrating that Lck is not required for TCR-mediated signaling in CD8$^+$ T$_M$ cells. Importantly, Lck independence was found at a range of peptide concentrations, showing that the enhanced sensitivity of T$_M$ cells to stimulation is also independent of Lck [52]. In contrast, CD8$^+$ T$_N$ cells were completely dependent on Lck for their activation and differentiation. It should be noted that an Lck-related Src-family tyrosine kinase, Fyn, can partially substitute for Lck during T-cell development [53], and requirements for Fyn in CD8$^+$ T$_M$ cell signaling remain to be tested.

In summary, although it appears that surface TCR levels are the same on T$_N$ and T$_M$ cells [50, 51], there are data to suggest that CD8$^+$ T$_M$ cells may have proximal TCR-associated molecules in a 'response-ready' configuration prior to stimulation, and/or may short-circuit some of the pathways used by T$_N$ cells. Given the role of the TCR as the major signal initiator for T cells, especially in the context of pathogenic infection [11, 54], it is possible that rapid relay of TCR-mediated signaling events is sufficient to explain the enhanced functional capacity of T$_M$ cells. However, several lines of evidence now support the idea that the 'ready-to-respond' state of resting CD8$^+$

T_M cells also involves TCR-distal mechanisms, including distinct chromatin conformations and mRNA expression profiles from those of their T_N precursors.

Chromatin remodeling

The evidence that proximal TCR signaling relays are not wholly responsible for the enhanced functional capacity of $CD8^+$ T_M cells was provided by Shen and colleagues [55], who investigated the mechanistic basis for the dysfunctional phenotype of $CD8^+$ T_M cells generated in the absence of $CD4^+$ T cell help. Initially, studies by the Shen and Bevan groups showed that such 'unhelped' $CD8^+$ T_M cells were defective in their ability to control bacterial replication following secondary infections [31, 32]. Northrop *et al*. showed that this was associated with decreased per cell production of IFNγ by unhelped T_M cells, as compared to their helped counterparts. This was true for both polyclonal and monoclonal (TCR Tg) $CD8^+$ T_M cells, the latter indicating that selection for lower affinity clones could not explain the defect in the unhelped environment. Most importantly, this defect in IFNγ production was also observed when a chemical stimulation protocol was used that bypassed proximal TCR signaling events [55].

An alternative explanation for the inability of unhelped $CD8^+$ T_M cells to robustly upregulate IFNγ production was that the IFNγ locus was in a 'closed' conformation in these cells, and not easily accessible to the transcriptional machinery. To test this hypothesis, two chromatin modifications were analyzed in $CD8^+$ T cells – DNA methylation at CpG dinucleotides (whose presence correlates with a closed chromatin conformation) and di-acetylation of histone H3 (whose presence is associated with an 'open' chromatin conformation). In $CD4^+$ T-cell-sufficient wild-type mice, CpG sites within regulatory regions of *Ifng* were heavily methylated in T_N cells. This methylation was dramatically lower in effector cells present at the peak of infection, and this hypomethylated state was maintained in resting T_M cells. However, CpG methylation levels at *Ifng* were equivalent between helped and unhelped $CD8^+$ T_M cells. In contrast, levels of di-acetylated histone H3 (diAcH3) were dramatically lower in unhelped T_M cells as compared to their helped counterparts. This led the authors to conclude that the failure of unhelped T_M cells to rapidly upregulate IFNγ was due, at least in part, to their inability to acquire and/or maintain appropriate levels of histone acetylation at the IFNγ locus.

A pattern of CpG hypermethylation at the IFNγ locus in $CD8^+$ T_N cells and hypomethylation in T_M cells was consistently observed in several reports, with studies in infection models confirming results originally obtained using naturally occurring polyclonal ($CD44^{hi}$) $CD8^+$ T_M cells [56, 57]. Specifically, a decade ago, Kelso and colleagues reported CpG hypomethylation within *Ifng* regulatory regions in $CD44^{hi}$ $CD8^+$ T_M cells. Conversely, polyclonal $CD44^{lo}$ $CD8^+$ T_N cells were hypermethylated at the same sites. Notably, they also demonstrated the heritability of these CpG patterns in the clonal progeny of T_M cells that had been isolated and cultured at a single cell level [57, 58]. Thus, multiple studies support the idea that the IFNγ locus attains a more open chromatin conformation during $CD8^+$ T-cell differentiation that is maintained in the long-lived population of T_M cells.

Importantly, the few studies that have compared human $CD8^+$ T_N and T_M cells have found similar patterns of chromatin modifications to those reported in murine models. Weng and colleagues compared levels of acetylated histone H3 (K9) at effector cytokine and chemokine loci [59]. They found that higher mRNA levels in stimulated T_M than T_N cells correlated with higher levels of $H3K9^{Ac}$ in the T_M cells. Given the well-known association of $H3K9^{Ac}$ with active transcription [60], this result was not very surprising. Interestingly though, at some cytokine loci, the $H3K9^{Ac}$ level was also higher in T_M than in T_N cells analyzed immediately *ex vivo*, when differences in cytokine mRNA levels between the two cell types were not detected, suggesting that these loci were 'poised' for activation in T_M cells. However, it should be noted that the authors did not provide compelling evidence for a strong correlation of resting histone acetylation levels with activation-induced transcript induction, as mRNA levels in activated cells were not measured until 72 h post-stimulation. Given the potential for up to 1 000-fold increases in cytokine mRNA levels within 4-8 h of T_M cell stimulation [56], the idea that such histone modifications are indicative of loci that are poised for 'rapid' expression remains to be tested.

More recently, Weng and colleagues have focused on mapping histone methylation levels in human $CD8^+$ T_N and T_M cells. Using chromatin immunoprecipitation combined with genome-wide DNA sequencing (ChIP-seq), they mapped the density of two histone H3 modifications: tri-methylation of lysine 4 (a mark associated with open chromatin) and tri-methylation of lysine 27 (a mark of closed chromatin) [61]. Within this rich data set, two observations stand out. First, a positive correlation between the H3K4me3 abundance of a locus and its mRNA level, which has been observed for other cell types, was confirmed for $CD8^+$ T cells on a genome-wide basis. Likewise, a negative correlation between the H3K27me3 abundance and mRNA expression level of a locus was also confirmed. Second, they found enriched H3K4me3 at many 'poised' loci in resting T_M cells (loci

whose expression is rapidly induced following T_M-cell stimulation). This observation builds on their previous reports, which found abundant di-acetylation of histone H3 at poised loci in $CD8^+$ T_M cells [59, 62], and suggests that H3K4me3 and diAcH3 may act cooperatively in keeping this 'poised' subset of loci open and accessible to the transcriptional machinery in resting T_M cells. In addition, among the actively transcribed genes in T_M cells, they found some depleted of diAcH3, but enriched for H3K4me3, and others with the opposite pattern (depleted of H3K4me3 and enriched for diAcH3), providing the first evidence for a division of labor among different modifications in $CD8^+$ T_M cells. Given this solid foundation, future studies using the powerful ChIP-seq technique should map the localization and abundance of diAcH3 (and other marks of open chromatin) in $CD8^+$ T_M cells, and address the correlation of these marks with silent, actively transcribed and poised loci. Such studies should strengthen the correlations found thus far and build a more detailed model of histone modification patterns in resting T_M cells, facilitating investigations into chromatin-based mechanisms underlying T_M cells' rapid recall ability.

Together, these studies suggest that development of rapid recall ability by $CD8^+$ T_M cells is associated with the acquisition and maintenance of distinct chromatin modification patterns at effector cytokine and chemokine loci. This work also highlights the fact that, to date, histone modifications in $CD8^+$ T cells have been mapped primarily at loci encoding effector molecules (Table 1), leaving open the question of whether loci encoding regulators of other processes, such as proliferation, migration, energy metabolism and/or biosynthetic pathways, are marked by distinct histone modifications in T_N and T_M

Table 1 Histone modifications at specific loci in resting $CD8^+$ T_M vs. T_N

					Specific enrichment for target region by ChIP[2]			
Cell type	Gene	Molecular function[1]	Modification	Region	T_M	T_N	T_E	REF
Human peripheral blood T_N: CD8+ CD45RA+ T_M: CD8+ CD45RA–	Ccr5	Chemokine receptor	Acetylated histone H3 (K9)	Promoters	++++	++	n.d.	[60]
	Klrb1	C-type lectin			++++	+	n.d.	
	Csf2	Cytokine			++	+	n.d.	
	Ifng	Cytokine			++	+	n.d.	
	IL2	Cytokine			++	+	n.d.	
	IL2ra	Cytokine receptor			++	+	n.d.	
	Tnfa	Cytokine			++++++++	+++	n.d.	
	Smarca1	Putative ATP-dependent chromatin remodeler			++++	++++	n.d.	
	Rab28	Putative Rab GTPase			+++++	+++++	n.d.	
	IL18r1	Cytokine receptor			+	+	n.d.	
Human peripheral blood T_N: CD8+ CD45RA+ T_M: CD8+ CD45RA–	Eomes	Transcription factor	Acetylated histone H3 (K9)	Promoter + 1st exon, averaged	++	+	n.d.	[63]
	Prf1	Pore-forming protein		Promoter	++	+	n.d.	
	Gzmb	Serine protease		Promoter + 1st exon, averaged	++	+	n.d.	
Human peripheral blood T_N: CD8+ 45RA+ CD62L+ T_{CM}: CD8+ 45RA–62L+ T_{EM}: CD8+ 45RA–62L–	Genome-wide ChIP-seq		Tri-methylated histone H3 (K4)	ORF + 1 Kb upstream of TSS were reported	See REF for specific loci			[62]
	Genome-wide ChIP-seq		Tri-methylated histone H3 (K27)					
Murine, TCR Tg T_N: P14 mouse, *ex vivo* T_E + T_M: P14 A.T., LCMV infected[3]	Ifng	Cytokine	Di-acetylated histone H3 (K9K14)	Promoter	+++++	++	++++	[56]
	Ifng	Cytokine		1st intron enhancer	++++++++	+	++++++++	
	IL2	Cytokine		Promoter	++	++	++	
	Cd3e	TCR signaling complex		Promoter	++	++	++	

[1]Molecular functions were obtained from the NCBI Gene database.
[2]Plus signs are used to symbolize the relative abundance of histone marks at the target regions between different cell types ("specific enrichment" refers to the relative amount of target DNA from a ChIP using an antihistone Ab vs. a negative control antibody).
[3]For these experiments, T_E and T_M were generated by adoptive transfer (A.T.) of P14 cells to congenic B6 recipients, who were then immunized with LCMV Armstrong. T_E were analyzed at day 8 post-infection and T_M were analyzed > 45 days post-infection.
Abbreviations: ORF = open reading frame; TSS = transcription start site; n.d. = not determined

cells. Furthermore, given the aberrant chromatin modifications found in $CD8^+$ T_M cells primed in the absence of $CD4^+$ T cell help, which are impaired in rapid recall ability, identification of the full spectrum of epigenetic differences between T_N and T_M cells, both functional and dysfunctional, in addition to the transcription factors and enzymes involved in regulating these modifications, should yield a much more detailed understanding of how the T_M 'ready-to-respond' state is maintained, and perhaps allow for its therapeutic manipulation.

Transcript profiles

Considering the role that chromatin modifications have in regulating transcription, their characterization should provide insight into studies that have identified the distinct mRNA expression profiles of resting $CD8^+$ T_N and T_M cells. For instance, in the first comprehensive study of the gene expression profile of $CD8^+$ T_M cells generated after acute viral infection, Kaech *et al*. [37] found higher expression of factors regulating migration, TCR signaling, cell cycle progression and T-cell effector functions in resting T_M than in resting T_N cells. Specifically, resting T_M cells contained ~two-fold higher transcript levels of the proximal TCR signaling molecules Fyn and Lck. Though Tewari *et al.* [52] later showed that virus-specific $CD8^+$ T_M signaling can be independent of Lck, these results suggest that Fyn may be used by T_M cells instead. It was also shown that resting $CD8^+$ T_M cells contained higher transcript levels of several cyclins regulating the G1 to S transition, as well as the G2/M cyclin B1. Given that the virus-specific T_M cell population contains a very small population of cycling cells at any given time (~1-3%), it was possible that the expression of S and G2/M cyclins was from these 'contaminating' dividing cells within the total T_M cell population. However, in a later study, the same group purified 'resting' T_M cells (in G1) and confirmed elevated expression of the G2/M cyclin B1, indicating that T_M cells may have a lower threshold for progression into the cell cycle following stimulation [63].

It should be noted that a similar comprehensive transcriptional profiling study was undertaken by Goldrath *et al*. [27], using $CD8^+$ T_M cells generated by LIP. Specifically, naïve TCR Tg (OT-I; specific for a peptide derived from ovalbumin) cells were adoptively transferred to partially irradiated congenic hosts and T_M cells were allowed to develop over 40 or 115 days. At the same time, the authors generated OT-I T_M cells by acute pathogenic infection, and identified their unique expression signature, as compared to their T_N and T_E cell precursors. Notably, the overwhelming majority of these pathogen-driven T_M-cell signature genes (95%) were also upregulated in the T_M cells generated by LIP. Furthermore, the authors were unable to identify any of the genes induced during LIP-driven T_M-cell differentiation that were not also induced in pathogen-driven T_M-cell differentiation [27]. The major distinction between $CD8^+$ T_M cells generated by LIP and pathogen-driven T_M cells was the level of gene induction that occurred at early times post-stimulation (< 7 days), which was lower for $CD8^+$ T cells that underwent LIP, especially for genes encoding effector molecules. Nevertheless, multiple comprehensive whole genome analyses of murine T-cell populations have shown that the 'expression signatures' of resting $CD8^+$ T_N and T_M cells are not identical, independently of whether the T_M cells were generated by infection or LIP.

Post-transcriptional regulation of effector molecules

As previously mentioned, it is likely that the functional capacity of $CD8^+$ T_M cells is regulated at multiple levels. Although the maintenance of an 'open' chromatin conformation at effector cytokine loci in resting T_M cells has been discussed as an explanation for their robust, stimulation-induced cytokine secretion, it is likely that post-transcriptional mechanisms also contribute. Indeed, one example of this regulation of effector molecules in T_M cells involves the chemokine RANTES (CCL5). In a series of studies, Marvel and colleagues [64] showed that the level of RANTES mRNA was ~five-fold higher in resting $CD8^+$ T_M cells than in T_N cells. This correlated with rapid secretion of RANTES from T_M cells, but not from T_N cells following stimulation (detectable within 20 min after *in vitro* stimulation of T_M cells). Importantly, T_M cell secretion of RANTES was abrogated when translation was inhibited by cycloheximide, but was unaffected when transcription was inhibited by actinomycin D, indicating that stimulation-induced transcriptional upregulation was not responsible for the rapid RANTES secretion. This was in contrast to IFNγ, which was highly upregulated early following stimulation of T_M cells in a manner dependent on transcription [64]. Whether other chemokines (and/or cytokines) are regulated in a manner similar to RANTES remains to be determined; nonetheless, this example indicates that immediate expression of effector molecules by stimulated T_M cells can be dependent on post-transcriptional mechanisms.

Conclusions

In conclusion, though rapid recall ability is a defining characteristic of $CD8^+$ T_M cells, we are only beginning to understand how it is acquired, maintained and executed. In the absence of stimulation, when compared to their T_N counterparts, there are several distinct properties of T_M cells that may contribute to their enhanced respon-

siveness. These include T_M cells' higher level of 'active' (phosphorylated) TCR-proximal signaling components, acquisition of an 'open' chromatin conformation at cytokine and chemokine loci and maintenance of pools of chemokine mRNA that can be rapidly translated following stimulation. In addition, mRNA profiling studies comparing resting T_N and T_M cells have identified differences in their levels of molecules involved in TCR signaling, migration, proliferation and metabolism. However, the extent to which these transcriptional differences explain the unique properties of resting T_M cells (homeostatic proliferation and tissue migration), stimulated T_M cells (enhanced proliferation, cytotoxicity and secretion of effector molecules) or both, is not clear. Thus, future studies that identify specific key regulators of T_M cells' rapid recall ability are warranted, and should greatly advance our understanding of T_M cell biology.

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