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Influence of time and number of antigen encounters on memory CD8 T cell development

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Abstract CD8 T cells are an important part of the adaptive immune system providing protection against intracellular bacteria, viruses, and protozoa. After infection and/or vaccination, increased numbers of antigen-specific CD8 T cells remain as a memory population that is capable of responding and providing enhanced protection during reinfection. Experimental studies indicate that while memory CD8 T cells can be maintained for great lengths of time, their properties change with time after infection and/or vaccination. However, the full scope of these changes and what effects they have on memory CD8 T cell function remain unknown. In addition, memory CD8 T cells can encounter antigen multiple times through either reinfection or prime-boost vaccine strategies designed to increase numbers of protective memory CD8 T cells. Importantly, recent studies suggest that memory CD8 T cell development following infection and/or vaccination is influenced by the number of times they have encountered cognate antigen. Since protection offered by memory CD8 T cells in response to infection depends on both the numbers and quality (functional characteristics) at the time of pathogen re-encounter, a thorough understanding of how time and antigen stimulation history impacts memory CD8 T cell properties is critical for the design of vaccines aimed at establishing populations of long-lived, protective memory CD8 T cells.

Keywords CD8 T cells · Memory · Vaccination · Survival · Prime-boost · Pathogens

Introduction

CD8 T cells play a critical role in combating infections caused by intracellular pathogens, including viruses, certain bacteria, and protozoan parasites [1]. The number of naïve CD8 T cells that bear T cell receptors (TCRs) recognizing an individual pathogen-derived epitope is low. Recent tetramer enrichment experiments have indicated that these numbers range from 10 s to at most 1,000 cells in laboratory mice [2, 3], numbers far too low to provide protection to the host from a rapidly spreading infection. However, during infection, CD8 T cells mount a pathogen-specific response that is initiated when dendritic cells (DCs) capture foreign antigen (Ag), migrate to draining lymph nodes, and present pathogen-derived Ags to naïve CD8 T cells [4]. Interactions with Ag-presenting DCs in the lymph nodes provide signals 1 (TCR stimulation), 2 (co-stimulation), and 3 (inflammatory cytokines) to naïve CD8 T cells initiating a period of robust proliferation where numbers of Ag-specific effector CD8 T cells increase in numbers by as much as 50,000-fold over a period of 7–8 days [5, 6]. During the vigorous expansion phase, effector CD8 T cells undergo a period of differentiation, acquiring the ability to migrate to infected tissues, to produce inflammatory cytokines including IFN-γ and TNF-α, and to lyse infected cells, responses which help to eliminate the invading pathogen [7]. Following the expansion phase, CD8 T cells undergo a period of programed contraction where 90–98 % of the Ag-specific CD8 T cells are eliminated through apoptosis [8]. The pool of

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cells that remain following contraction constitutes the memory CD8 T cell population that is capable of responding and providing enhanced protection during reinfection with the same pathogen.

What characteristics of memory CD8 T cells account for increased protection compared to naïve CD8 T cells? To answer this question, one must take into account (1) differences in the numbers of naïve and memory CD8 T cells present in an intact host, and (2) potential functional differences between naïve and memory CD8 T cells when these cells are compared on a per-cell basis.

Perhaps, the biggest difference is seen at the population level, as the precursor frequency of Ag-specific cells is much greater for the memory than the naïve CD8 T cell population of the same Ag specificity. This large pool of memory CD8 T cells is also capable of robust secondary expansion following reinfection resulting in a large population of secondary effector CD8 T cells that is able to quickly counter invading microbes [9, 10].

On the cellular level, memory CD8 T cells may begin to proliferate more quickly than naïve CD8 T cells as they accumulate pre-activated cyclin D3/CDK6 complexes in the cytoplasm and contain lower levels of p27kip inhibitors allowing them to remain poised in the late G1 phase of the cell cycle [11, 12]. However, recent work questioned these assumptions showing that naïve CD8 T cells have a lower antigen threshold requirement for cell cycle entry than central memory CD8 T cells. Central memory CD8 T cells did not activate Zap70, induce cMyc expression, or degrade p27 in response to Ag levels that triggered these responses in naïve CD8 T cells [13].

Additionally, following primary infection, the memory CD8 T cell population includes cells that reside in peripheral tissues where they can encounter infection faster than naïve CD8 T cells localized predominantly in secondary lymphoid organs [14, 15]. In contrast to naïve cells, memory CD8 T cells also can quickly relocate to peripheral tissues in response to inflammation associated with infection [16]. Core 2 *O*-glycan expression on memory CD8 T cells recently was shown to be altered in response to IL-15, facilitating interactions with P- and E-selectins and migration into inflamed tissues during infection [17].

Finally, memory CD8 T cells are able to execute effector functions, such as cytokine production and cytolysis, more quickly than naïve CD8 T cells, requiring only a brief 5–6-h period of stimulation to elaborate effector functions [18–20]. Faster effector responses by memory CD8 T cells are facilitated by preformed stores of cytolytic molecules, enhanced TCR-proximal signaling, and permanent and heritable chromatin remodeling allowing for easier access to gene transcription machinery [21–24]. All together, these characteristics comprise the hallmark attributes of protective memory CD8 T cells.

While memory CD8 T cells possess different properties than naïve CD8 T cells, the memory population is comprised of a heterogeneous group of cells differing from one another in phenotype and functional characteristics [25, 26]. Subsets of memory cells have been described based on expression of surface molecules that confer functionality, including the ability to survive, to traffic, and to localize within tissues. Classically, memory CD8 T cells have been divided into two subsets, effector memory (T_{em}) and central memory (T_{cm}). T_{cm} express chemokine receptor 7 (CCR7) and L-selectin (CD62L), which allow efficient trafficking to lymph nodes, while T_{em} cells do not express these molecules and localize more efficiently to peripheral tissues [27, 28]. While T_{em} and T_{cm} are both efficient at producing IFN-γ and TNF-α, T_{cm} are better equipped to produce IL-2, have a greater capacity to persist in the host, and undergo higher magnitudes of proliferative expansion upon Ag re-encounter [29]. Therefore, T_{cm} may be important in controlling prolonged and/or systemic infection. In contrast, localized infections in peripheral organs may be better handled by another subset of memory CD8 T cells termed tissue-resident memory cells (T_{rm}). T_{rm} cells express the α-chain of $\alpha_E(CD103)\beta_7$ integrin and do not recirculate, but are instead permanent and long-lasting residents of peripheral tissues [15]. T_{rm} populations have been shown to provide protection in the skin and female genital tract in response to infection with herpes simplex virus [30, 31]. They can also stimulate localized immune responses by producing cytokines and chemokines including IFN-γ and CXCL9 that attract additional immune cells to the site of infection [32].

Heterogeneity within the memory CD8 T cell population is likely much broader than the T_{em}, T_{cm}, and T_{rm} classifications. Memory CD8 T cells have been divided into additional subsets based on expression of a member of the TNF-receptor family (CD27) and a glycosylated form of sialophorin (CD43) [33]. $CD27^{lo}CD43^{lo}$ populations display similar, but also unique patterns of expression of transcription factors and surface markers compared to T_{em} and T_{rm} subsets, and despite a reduced ability to proliferate, $CD27^{lo}CD43^{lo}$ cells provided better protection against infection with *Listeria monocytogenes* (*L. monocytogenes*) due to the ability to localize to the red pulp of the spleen [34]. Thus, successful vaccination strategies must take into account whether memory CD8 T cells of the appropriate quality will be generated to counter infection.

Another important consideration for the design of protective vaccines is that differentiation from an effector to memory CD8 T cell is a process that takes considerable time. Gene expression profiles of CD8 T cells continue to change during the transition from effector to memory cell, and a period of time is required for CD8 T cells to acquire characteristics of memory including the ability to

self-renew and proliferate in response to Ag [35]. However, the period of time required for memory differentiation is not fixed, and levels of inflammation present during the time of either infection or vaccination appear to strongly influence the size of effector CD8 T cell responses and the size of the resulting memory CD8 T cell pool, as well as the rate at which CD8 T cells acquire memory characteristics [36, 37]. The cytokines IL-2, IL-12, and type I interferons appear to be highly important in this regulation [38–42]. Recently, IL-12 and type I interferons were shown to regulate expression of the high-affinity subunit of the IL-2 receptor (CD25 or IL-2Rα). Sustained expression of CD25 due to signals transmitted by IL-12 and type I interferons increased sensitivity to IL-2 and allowed for extended division and increased accumulation of activated CD8 T cells [42]. Limiting the duration of bacterial infection through the antibiotic treatment (leading to lower levels of inflammation) resulted in the generation of CD8 T cells displaying memory characteristics, such as the ability to undergo vigorous secondary expansion and increased IL-2 production upon Ag re-encounter, within 2 weeks [43]. Additionally, vaccination using peptide-coated mature DCs that elicit low levels of inflammation resulted in the formation of memory CD8 T cells that could be boosted as soon as 4 days following DC priming [44, 45]. Administration of CpG to induce systemic inflammation reversed this accelerated memory differentiation after either DC immunization or antibiotic treatment [43–45]. Thus, inflammation can greatly impact numbers of memory CD8 T cells generated and the rate of programed memory development following infection and/or vaccination. This is an important consideration for the design of vaccines utilizing inflammation-inducing adjuvants.

Additional considerations for the design of protective vaccines include how memory CD8 T cell function changes with time after infection and with additional Ag encounters. Memory CD8 T cells are able to persist for great lengths of time after infection and/or vaccination [46], and an important and perhaps underappreciated consideration in vaccine design is that the properties of memory CD8 T cells continue to change with time after infection and/or vaccination. While this has been explored to some extent, a more thorough understanding of how time affects memory CD8 T cell properties is needed. Additionally, humans are often infected multiple times with the same or related pathogens, leading to the formation of memory CD8 T cell populations that have encountered their cognate Ag more than once. The numbers of memory CD8 T cells required to achieve protection from infection may be higher than can be achieved with a single immunization [47], and prime-boosting vaccination strategies designed to elicit large numbers of memory cells result in memory CD8 T cells that have encountered Ag multiple times [48]. While the numbers of memory CD8 T cells increase with additional Ag encounters, it has become clear that the properties of memory CD8 T cells are dependent upon Ag stimulation history. Together, changes in memory CD8 T cell properties with time after infection and/or vaccination and with additional Ag encounters constitute important considerations for effective vaccine development, and this will be the focus of the remainder of this review.

Maintenance and function of primary memory CD8 T cells with time after infection

Maintenance and longevity of primary CD8 T cell memory

While most vaccines are intended to prevent either seasonal illnesses or infections that may be encountered in the relatively near future, infection may not occur for long periods of time following the original vaccination. Thus, it is important to establish whether CD8 T cell memory that is formed after vaccination is maintained throughout life, and how the function of memory CD8 T cells changes over extended lengths of time. These questions are easier studied in animal models where re-exposure to infection can more easily be controlled. However, the durability of CD8 T cell memory following either infection or vaccination in humans has been studied in the context of several acute viral infections that only cause rare infections (measles virus), or are not endemic (vaccinia virus and yellow fever virus) [46, 49].

Unlike naïve cells, memory CD8 T cells do not require either TCR signaling or MHC class I molecules for long-term survival [50, 51]. Instead, memory CD8 T cells are maintained in vivo through a slow process of basal turnover that is dependent on the pro-survival cytokines IL-7 and IL-15 [52]. Decreased percentages of IL-7Rα deficient CD8 T cells survive and differentiate into memory cells following infection, and memory CD8 T cells generated in either IL-15 or IL-15Rα deficient mice slowly decline in numbers [53, 54]. In order to maintain stable numbers of memory CD8 T cells, basal turnover, which results in two daughter cells, must be balanced by an equivalent rate of cell death. Our recent work has indicated that basal turnover results in the formation of a subset of memory CD8 T cells termed T death intermediate memory (T_{DIM}), which are nonfunctional (i.e., are unable to produce cytokines or internalize TCRs following stimulation with Ag), are destined to die and presumably serve to keep numbers of memory CD8 T cells stable during basal turnover [55]. Thus, basal proliferation allows the memory CD8 T cell population to be maintained at relatively stable numbers for great lengths of time.

Homann et al. [56] examined the longevity of epitope-specific memory CD8 and CD4 T cell populations in mice following lymphocytic choriomeningitis virus (LCMV) infection. Numbers of memory CD4 and CD8 T cell populations were determined in peripheral blood (PBL) by ELISPOT and MHC class I or class II tetramers at various time points for over 900 days following LCMV infection. While numbers of Ag-specific memory CD4 T cells were found to decline with time after infection, numbers of each of the six analyzed epitope-specific memory CD8 T populations remained constant. Memory CD8 T cells were found to retain expression of the anti-apoptotic protein Bcl2 to a greater extent than memory CD4 T cells, potentially leading to an increased resistance to apoptosis. In agreement with the longevity of LCMV-specific CD8 T cell memory in mice, a recent report by Valkenburg et al. [57] examined memory CD8 T cell generation in response to infection with influenza virus. Detectable numbers of memory CD8 T cells specific for two different epitopes of influenza virus were detected by intracellular cytokine staining (ICS) greater than 22 months after infection with the H3N2 strain of influenza virus. Collectively, these and other studies indicate that primary CD8 T cell memory established early in life can be maintained for the life of the mouse.

While mice can be housed in specific pathogen-free facilities, humans are repeatedly exposed to multiple unrelated pathogens. Since the size of the memory CD8 T cell pool is thought to be limited by constraints of space and availability of survival factors such as IL-7 and IL-15, exposure to unrelated infections could lead to attrition of memory CD8 T cell populations. Schmidt et al. [58] found that the numbers of memory CD8 T cells specific for a malaria circumsporozoite protein were sharply reduced following infection with four unrelated bacteria and viruses. Additionally, Selin et al. [59] found that LCMV-specific memory CD8 T cells undergo substantial attrition in mice following one or more heterologous virus challenges. Further experiments by Varga et al. [60] determined that attrition of LCMV-specific memory CD8 T cells was independent from CD4 T cells, as the numbers of LCMV-specific CD4 T cells remained stable, while the numbers of memory CD8 T cells declined following heterologous challenges. However, a study by Vezys et al. [61] found that the memory CD8 T cell compartment can grow in size following sequential infections to allow maintenance of pre-existing memory populations. While they observed attrition of LCMV-specific memory CD8 T cells following a series of three heterologous infections with vesicular stomatitis virus (VSV) strains, the severity of attrition was more modest than indicated by earlier studies. However, these studies indicate that memory CD8 T cells in humans, who are repeatedly exposed to nonrelated infections, may decrease in number, which could lead to loss of CD8 T cell-mediated protection.

A number of studies, however, have indicated that memory CD8 T cells in humans are detectable for many years following either infection or vaccination. Ahmed and Akondy have reported that following a live virus vaccination against yellow fever (YFV-17D), memory CD8 T cells could be detected for decades following vaccination [62]. Similarly, peripheral blood mononuclear cells (PBMCs) from vaccinia virus-vaccinated individuals were able to lyse vaccinia virus-infected target cells 30 or more years after original vaccination [63]. Additionally, vaccinia virus-specific CD8 T cells persisted for long periods of time following natural exposure to smallpox or after vaccination [64, 65]. Detection of IFN-γ- and TNF-α-producing CD8 T cells by ELISPOT suggested that vaccinia virus-specific memory CD8 T cells declined with time after vaccination with a half-life between 8 and 15 years. However, 50 % of individuals receiving one vaccination possessed detectable CD8 T cell memory at least 20 years after vaccination, and at least one individual possessed memory CD8 T cells 75 years after vaccination [65]. Naniche et al. [66] have also reported that measles virus-specific CD8 T cells were detectable by ICS in individuals up to 34 years after vaccination. Thus, while the numbers of memory CD8 T cells may decline with time after infection and/or vaccination in human subjects, these cells can be maintained for the life of the host.

Changes in primary memory CD8 T cell properties with time after infection

Differentiation of memory CD8 T cells from the effector population requires time, and it appears that memory differentiation is a process that continues for great lengths of time following infection. While a limited number of experiments have examined the functional changes that occur in memory CD8 T cell populations with time after infection, this question remains largely underexplored (Fig. 1). This is an important unresolved question, as protection offered by memory CD8 T cells is based both on the quantity (numbers) and quality (functional ability) of these cells at the time of infection. Thus, changes in memory CD8 T cell function that occur with time after infection could directly impact their ability to provide protection from infection.

Expression of phenotypic markers including CD62L and CD27 has been shown to increase in the memory population with time after infection [67, 68]. Based on these markers, the memory population becomes comprised of primarily T_{cm} cells, suggesting that the overall function/responsiveness of memory CD8 T cells may change with time after infection. Jabbari et al. showed that the ability of

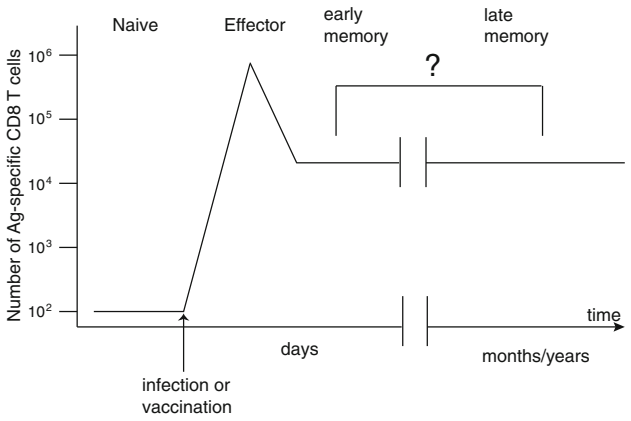

Fig. 1 The CD8 T cell response to infection or vaccination. Upon encountering antigen, naïve CD8 T cells differentiate into effector cells and undergo robust proliferative expansion in numbers over a period of several days. The effector response is followed by a period of contraction, and the cells remaining after contraction constitute the memory CD8 T cell pool. Memory CD8 T cells can be maintained for years; however, their properties change over the course of time. The full extent of changes that occur in memory CD8 T cells with time after infection is presently unknown

memory CD8 T cells to produce IL-2, a function that is better suited to T_{cm} compared to T_{em} cells, increased with time up to 80 days after infection with *L. monocytogenes*. T_{cm} also exhibit an increased ability to undergo proliferative expansion compared to T_{em} cells, and Roberts et al. [69] examined whether the ability of memory CD8 T cells to proliferate following Ag re-encounter changes with time after infection. In elegantly designed experiments, they examined proliferative potential of "aged" (12 months after infection) and "recent" (1 month after infection) Sendai virus-specific memory CD8 T cells. They found that "aged" memory proliferated to a greater extent than "recent" memory indicating that the ability of memory CD8 T cells to proliferate in response to Ag re-encounter increases with time after infection. Similarly, using TCR transgenic OT-I cells that recognize the $OVA_{257–264}$ epitope derived from chicken ovalbumin, we generated "early" memory (1 month) and "late" memory (8 months) after infection with *L. monocytogenes* expressing OVA and reported that the ability of memory CD8 T cells to proliferate in response to secondary Ag encounter increased with time after infection [10]. Importantly, higher proliferative potential but indistinguishable kinetics of secondary CD8 T cell responses generated from late versus early primary memory CD8 T cells leads to an increase in secondary memory CD8 T cell numbers, suggesting that "memory generation potential" of primary memory CD8 T cells is dependent on the age of the cells.

Thus, the limited amount of data on the functional changes that occur over time in the memory CD8 T cell population indicates that memory CD8 T cells regain expression of surface markers including CD27 and CD62L, have an increased capacity to produce IL-2, proliferate more robustly in response to Ag re-encounter, and have a greater memory generation potential. These data obtained in mice suggest the exceptional possibility that the memory CD8 T cell pool continues to differentiate for extended periods of time. The extent to which memory CD8 T cells change with time in humans is not known, but it would be interesting to determine whether the process of memory CD8 T cell differentiation continues for great lengths of time in long-lived humans. Additionally, how the functional changes that occur in memory CD8 T cells with time after infection affect their ability to confer protection from infection is largely unknown. The characteristics of memory CD8 T cells that confer protection to some pathogens may not lead to protection from others [70]. Thus, the ability of memory CD8 T cells to confer protection may increase with time for some infections and decrease with time for others.

Importantly, the success of booster immunization strategies may be dependent upon the changes in memory CD8 T cells that occur over the period of time between boosts. Recently, utilizing a vaccine strategy involving intravenous injection of cryopreserved *Plasmodium falciparum* sporozoites (PfSPZ), Seder et al. [71] reported that all human subjects receiving five injections were protected, while not all subjects receiving four or fewer injections were protected upon controlled human malaria infection. The authors indicated a number of potential reasons for the increased protection provided using the 5 dose immunization regimen including that these subjects had received the highest dose of PfSPZ and that there was an increased interval (7 weeks) between the fourth and fifth booster challenges. They argued that the increased length of time between the fourth and fifth dose may have led to greater numbers of CD8 T cells than would have been achieved using a shorter time frame between doses. Thus, changes that occur within the memory CD8 T cell population over periods of time between boosts may directly impact protection achieved through booster immunizations. For these reasons, a better understanding of the full spectrum of changes that occur in the memory CD8 T cell population with time and the implications of these changes for conferring protection from reinfection is needed. Ongoing work in our laboratory is examining this important knowledge gap.

Ag stimulation history influences memory CD8 T cell phenotype, function, and gene expression patterns

Models for studying multiple Ag encounters

Prime-boost protocols represent an attractive strategy for increasing the number of protective memory CD8 T cells,

and substantial research in the past several years has been devoted to understanding how the properties of memory CD8 T cells are affected by additional Ag encounters. There are two general models used for studying memory CD8 T cells that have encountered Ag multiple times, each with strengths and limitations.

The first model utilizes serial adoptive transfers (AT) of low numbers of TCR transgenic T cells or endogenous memory CD8 T cells into naïve mice (Fig. 2a). Transferred cells display a different allelic form of a surface protein (i.e., Thy1.1 or CD45.1) from the recipient host allowing for easy identification of the transferred cell population. This method allows for the detection of highly pure memory CD8 T cell populations with a precisely defined number of Ag encounters, as AT into naïve mice prevents rapid clearance of the pathogen, transfer of low numbers of memory CD8 T cells ensures complete recruitment of the transferred memory CD8 T cells, and identification of memory CD8 T cells based on allelic differences in surface marker expression excludes the contribution of newly recruited naïve CD8 T cells into the response [72].

The second model utilizes serial heterologous or homologous infections of intact mice and examination of endogenous memory populations. Using this method, resulting memory CD8 T cell populations likely are comprised of a heterogeneous population of cells that have encountered Ag a different number of times due to recruitment of new naïve precursors or incomplete recruitment of memory cells in ensuing responses. However, a recent study explored the capacity of the host to prime/activate existing memory CD8 T cells and suggested that most if not all of the existing memory CD8 T cells in repetitively infected hosts can respond to subsequent Ag encounter, although the number of memory CD8 T cells present at the time of re-challenge influences their subsequent differentiation and function [73]. Thus, while this model may not allow for the examination of highly pure memory populations with a defined Ag stimulation history, it may more accurately represent the memory CD8 T cell response as it occurs in intact hosts following repeated Ag encounters. While the models lead to some similar and some different conclusions regarding the impact of repeated Ag encounters on the properties of memory CD8 T cells, both models are necessary to gain a complete picture of the effects of additional encounters on CD8 T cell biology.

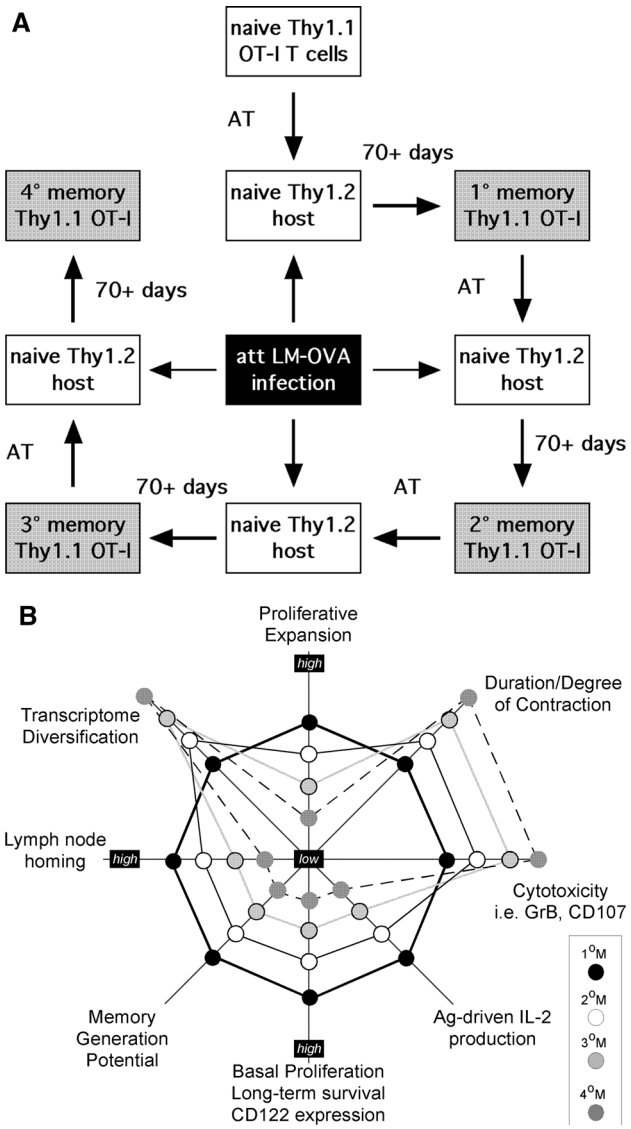

Fig. 2 The affects of multiple Ag encounters on CD8 T cell properties. **a** Model of serial adoptive transfers for the study of memory CD8 T cells that have encountered Ag multiple times. 1° memory is generated by adoptive transfer of physiological numbers of transgenic cells into naïve hosts, which are subsequently infected with a pathogen-expressing cognate antigen. *Low numbers* of memory cells are subsequently transferred into new naïve recipients followed by infection to generate a 2° memory population, while 1° memory is generated in a second group of mice as described above. This process is repeated until 1°, 2°, 3°, and 4° memory populations of the same time after last infection was generated differing only in the number of times they have encountered their cognate antigen. **b** Functional characteristics of memory CD8 T cells that have encountered Ag multiple (1–4) times. Functional abilities and characteristics are highest for memory populations at the outermost edges and progressively decrease for memory populations moving inward (i.e., proliferative expansion 1° > 2° > 3° > 4°)

Effects of additional Ag encounters on memory CD8 T cell properties

Microarray studies have shown that memory CD8 T cells have a unique gene expression profile compared to naïve and effector CD8 T cells [35]. Do gene expression profiles of memory CD8 T cell populations that have encountered Ag more than once differ from one another? We recently examined this question using the serial adoptive transfer

model to generate primary (1°), secondary (2°), tertiary (3°), and quaternary (4°) memory OT-I cells that differed only in the number of Ag encounters [72]. A group of genes representing a "memory core signature" were found to be differentially expressed in naive compared to memory CD8 T cells regardless of the number of Ag encounters. However, gene expression patterns among memory CD8 T cell populations that had encountered Ag 1–4 times differed from one another, and more than 700 genes were differentially regulated between 1° and 4° memory cells. Interestingly, the number of genes that were differentially expressed between memory populations and naive CD8 T cells increased with each additional Ag encounter indicating that repetitive Ag stimulation induces stepwise changes in gene expression patterns (Fig. 2b). Changes in gene expression after additional Ag encounters could have been due to altered subset (T_{em}/CD62L^{low}:T_{cm}/CD62L^{hi}) ratios of memory populations without major changes in gene expression within individual cells. Alternatively, repeated Ag encounter may have altered gene expression within cells of the same subset in ensuing memory CD8 T cell populations. However, gene set enrichment analysis (GSEA) performed on the list of genes that are up-regulated in T_{em} showed no progressive enrichment in "effector memory" associated genes in 2°, 3°, and 4° memory CD8 T cell populations, suggesting that each round of Ag stimulation further increases the complexity of memory CD8 T cell populations in a manner that may supersede current T_{em} and T_{cm} classifications. Additionally, expression of many genes in memory CD8 T cell populations continued to either increase (*Gzmb, Anxa1, Ccr5*) or decrease (*Actn1, Ccr7, Treml2*) in expression with additional Ag encounters indicating that functional differences may become magnified as memory CD8 T cells encounter Ag additional times. Genes that were differentially regulated with additional Ag encounters clustered into families of genes-regulating effector functions, signaling, migration, adhesion, cell cycling, and apoptosis. This list provides a starting point for studying the multitude of functional changes that likely occur after additional Ag encounters. Additionally, the expression levels of several transcription factors including *Eomes, Prdm1* (Blimp-1), *Tbx21* (T-bet), and *Tcf7* (TCF-1) either progressively increased or decreased with additional Ag encounters. An understanding of how these transcription factors control expression of genes important for memory CD8 T cell function and whether they can be manipulated to generate highly functional and protective CD8 T cells represents an important area of further study.

As discussed previously, with time after infection, primary memory CD8 T cells gradually reacquire expression of surface molecules, including CD127, CD62L, CD27, and CD122 [74]. Interestingly, in both models of serial adoptive transfer and serial heterologous boosting, expression of these surface molecules in populations of memory CD8 T cells further decreases with each additional Ag encounter (Fig. 2b) [67, 68, 72]. Furthermore, the period of time required for re-expression of these molecules in the memory CD8 T cell population increases with subsequent Ag encounters [67, 68]. Consistent with their low expression of CD62L, localization of memory CD8 T cells in lymph nodes decreases with additional Ag encounters, while their localization in peripheral tissues increases with additional Ag encounters (Fig. 2b) [67, 68, 72]. This is an important consideration for vaccine design as control of infection may be better suited to memory CD8 T cells that are either peripherally or centrally localized depending on the nature of the pathogen.

Ag stimulation history also appears to have an impact on proliferation and memory generation following Ag re-encounter. Using the serial adoptive transfer model, we reported that the ability of memory CD8 T cells to undergo proliferative expansion following infection decreased with subsequent Ag encounters from 1° to 4° memory (Fig. 2b) [72]. Additionally, while the period of contraction was delayed for 2°, 3°, and 4° memory, contraction among these populations was eventually more vigorous than 1° memory. Each subsequent Ag encounter led to reduced expression of CD122 (IL-15Rβ), reduced responsiveness to IL-15, and decreased basal turnover of the memory population (Fig. 2b) [67, 72]. This suggests that with subsequent Ag encounters, memory CD8 T cells may have a reduced ability to be maintained through basal proliferation. However, a recent report by Fraser et al. [73] reported that the phenotype and function of memory CD8 T cell populations are dependent upon the number of memory cells present during Ag re-encounter. Utilizing the serial heterologous infection model or by transferring 2° memory cells into 2° immune mice, they found that when high numbers of memory cells participated in the immune response, acquisition of CD8 T cell memory characteristics following infection was rapid. The ensuing memory population quickly reacquired expression of CD127 and was able to robustly proliferate upon Ag re-encounter. Bioenergetics studies indicated that the 3° memory populations generated within the context of a large immune response possessed increased mitochondrial function and increased respiratory capacity may account for the high-proliferative potential of resulting 3° memory CD8 T cells using this system. Thus, in intact subjects in whom a large number of memory cells participate in the immune response, functional abilities of memory CD8 T cells may be preserved following recurrent Ag encounters.

CD8 T cells combat infection through the production of cytokines to stimulate the immune response and by killing infected target cells primarily through secretion of

perforins and granzymes. The evidence suggests that while the ability of memory CD8 T cells to produce IFN-γ and TNF-α is unaffected by the number of Ag encounters, the ability to produce IL-2 decreases with additional Ag encounters, while preformed stores of granzymes and ability to kill infected target cells increase with additional Ag encounters [67, 68, 72]. The enhanced ability to kill infected cells would lead one to believe that memory CD8 T cells that have encountered Ag multiple times would be more protective. However, because different pathogens utilize distinct anatomical niches in which to replicate, perhaps it is not surprising that memory CD8 T cells that have encountered Ag multiple times are more protective than 1° memory in some instances and less protective in others. 2° memory CD8 T cells generated through serial adoptive transfers were found to provide enhanced protection compared to an equal number of 1° memory cells following acute systemic infection with either *L. monocytogenes* or vaccinia virus (VacV) despite decreased ability to undergo proliferative expansion [67, 70]. However, 1° memory cells were found to be more protective than an equal number of 2° memory CD8 T cells following chronic infection with LCMV-clone 13. Because clone 13 infection is more efficiently controlled by CD8 T cells that are able to localize to the lymph nodes, differences in anatomical location may account for decreased protection of 2° memory cells following this chronic infection. An additional explanation is that memory CD8 T cells that have encountered Ag multiple times share a genomic signature with exhausted memory cells [72], and 2° memory CD8 T cells responding to clone 13 infection displayed increased expression of molecules expressed by exhausted CD8 T cells, including PD-1, 2B4, LAG-3, and CD160, compared to 1° memory CD8 T cells responding to clone 13. However, as would be indicated by an increased ability to undergo proliferative expansion, 2° memory CD8 T cells generated through heterologous prime-boosting of individual mice were at least as protective as 1° memory cells after infection with clone 13 [73]. As the above data illustrates, careful consideration must be made regarding the nature of the pathogen and the properties of memory CD8 T cells generated through prime boost for the design of effective vaccine strategies.

As discussed previously, the length of time required for memory CD8 T cells to express phenotypic markers including CD62L and CD27 increases with additional Ag encounters. That expression of these markers increases with time indicates that as with primary memory, functions of memory CD8 T cells that have encountered Ag multiple times, such as maintenance, cytokine production, and the ability to proliferate and generate memory, may change with time after infection. On the other hand, slower reacquisition of expression of these markers suggests that changes in properties of memory populations that have encountered Ag multiple times may occur at a slower rate than in primary memory CD8 T cell populations. This adds an additional layer of complexity to considerations of vaccine design utilizing prime boost strategies, and an understanding of the changes that occur with time after infection in memory CD8 T cell populations that have seen Ag multiple times needs to be determined. We are currently exploring this important question in ongoing experiments in our laboratory.

Conclusion

Since protection offered by memory CD8 T cells is based on the quantity and quality of memory cells present at the time of infection, a thorough understanding of the parameters that affect memory CD8 T cell function is necessary. Because reinfection may not occur for great lengths of time following either initial vaccination or primary infection, understanding how the function of memory CD8 T cells changes with time after either infection or vaccination represents a critical knowledge gap. Additionally, because humans are often repeatedly exposed to the same pathogen and because prime–boost regimens are an attractive strategy to increase the numbers of memory CD8 T cells, understanding how the properties of memory CD8 T cells are affected by additional Ag exposures is necessary. Furthermore, the effects of time on the properties of memory CD8 T cells that have encountered Ag multiple times appear to occur at different rates compared to primary memory. How the effects of time compared between primary memory and memory CD8 T cells that have encountered Ag more than once represents an additional knowledge gap in the field. Addressing these questions may allow us to develop more effective vaccine strategies designed to elicit the high numbers of functional and protective memory CD8 T cells.

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