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REJUVENATION OF T CELL MEMORY

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Abstract Memory $CD8^+$ T cells generated during an immune response are long-lived and self-renewing, offering enhanced host protection against re-infection. However, how an antigen-specific population of memory T cells is maintained throughout repetitive infections over potentially a lifetime is not known. Here we review the generation and maintenance of antigen-specific $CD8^+$ T cells and introduce our recent data showing dynamic turnover of an antigen-specific memory T cell population during repeated antigen challenge in vivo. We demonstrated that a primary response potentially occurs upon every recall response and find that the skewed T-cell receptor (TCR) repertoire of pre-existing memory T cells is partly corrected by diversity in a newly formed (primary) population. Importantly, memory T cells generated in a more recent antigen encounter expand more vigorously in a subsequent recall response. A primary response during re-challenge therefore restores both the TCR diversity and proliferative potential of the memory T cell population. These findings indicate that memory T cell populations evolve over multiple challenges, favoring memory T cells generated in more recent encounters, and suggest that these primary populations have essential roles in the perpetuation of antigen-specific T cell populations.

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Key words: memory $CD8^+$ T cell; primary response; maintenance

Introduction

Immunity is said to have a memory for most invading agents encountered before, because a second encounter with the same agent prompts a rapid and vigorous response. This is called immunological memory which leads to a perception that an individual is immune to a particular agent. This is the basis for the vaccination. This immunological memory was documented first in the record of the plague of Athens in 430 B.C. (ancient Greek era), describing that the "same man was never attacked twice". It is noteworthy that people acknowledged "immunological memory" long before the discovery of either viruses or of the immune system. The earliest attempts to put this into practice were made in China and India around 900 A.D. The pustular materials from smallpox patients were inoculated into healthy people, which generally resulted in a milder disease than a natural smallpox infection. Then in 1796, Edward Jenner has opened the fields of immunology or vaccination (from the Latin word, vacca, for cow) by inoculating subjects with cowpox exudates to protect them from smallpox infection.

Immunological memory is an exclusive property of the "adaptive" or "acquired" immune system. Notably, antigen-specific clones of T cell receptor (TCR) $\alpha\beta$ expressing T cells and B cells with help from T cells proliferate and differentiate in response to a primary infection and remain in the host at relatively high frequencies after resolution of the infection. In this paper, we will focus attention on the memory T cell response to the repetitive infection and discuss the new insight into the mechanism of the life-long memory $CD8^+$ T cell response.

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Development of T cell memory

Memory is a dynamic state. Much of our current knowledge on the development of T cell memory has come from the study of acute viral infection. The dynamic response of antigen specific $CD8^+$T cells to an acute viral infection has now been resolved at cellular and molecular levels in mice and humans. Once T cells encounter their cognate antigens, antigen-specific T cells differentiate from naïve cells into memory cells in the primary response. It is now accepted that they pass through three different stages (Figure 1, upper panel). When naïve T cells are primed in the lymphoid tissues, they start to clonally expand and differentiate into effector T cells (the 'expansion' phase). These activated effector T cells leave the lymphoid tissues and recruit into the local site (s) of infection to resolve it by their abilities to secrete inflammatory cytokines and kill infected cells. Generally acute infection can be resolved within several days. The majority of effector T cells die by apoptosis after the peak of response (the 'contraction' phase). Some antigen-specific T cells survived and are maintained for long periods of time (the 'memory' phase).

When the same antigen launches another attack, the memory T cells ensure that a recall response (also called as secondary or memory response) to foreign antigen is greater in magnitude and faster than a naïve T cell response. This enhanced response results in part through the increased frequency, proliferative responsiveness, and effector function acquisition of memory T cells compared to naïve T cells. The concept of Vaccination or Immunization is based on immunological memory.

As antigen specific T cells chronologically pass through these stages, their gene expression profile is reprogrammed by alterations in chromatin structure and the profile of active transcription factors, leading to differential expression of surface (including adhesion/homing) and effector (including cytokines) molecules. This enables us to distinguish different subpopulations with different functional properties among antigen specific T cells to a single antigen. Even within memory phase, different phenotypic subpopulations of memory T cells have been known to exist. Recently, a model of 'central memory' (T_{CM}) and 'effector memory' (T_{EM}) T cells has been proposed, based on the expression of adhesion/homing molecule CD62L (L-selectin) and chemokine receptor CCR7 (Figure 1, lower panel). This model introduced the direct linkage between anatomical distribution and protective/proliferative capacities of memory T cell subpopulations.

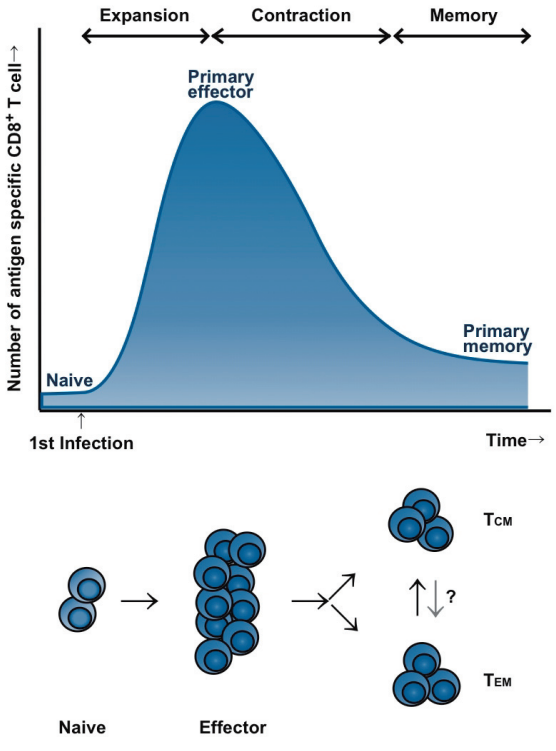

Figure 1 Development and subpopulation of memory $CD8^+$ T cells. (Upper panel) Chronological three major stages of antigen-specific $CD8^+$ T cell upon typical acute infection. (Lower panel) Memory T cell population can be divided by T_{CM} and T_{EM} based on CD62L and CCR7 expression.

Maintenance of memory T cells - Are memory T cells immortal?

Memory T cells can persist for years to a lifetime in humans through homeostatic proliferation - a feature reminiscent of stem cells - and therefore offer increased protection against foreign antigen long after an initial immune response has occurred[1-7]. Thus, a memory T cell population, once established, is generally thought to expand and contract clonally upon antigen re-challenge. However, it is not clear whether memory T cells can undergo such rounds of extensive proliferation indefinitely.

Although a recall T cell response consists mostly of pre-existing (secondary) $CD8^+$ T cells, some naïve (primary) $CD8^+$ T cells are newly primed by antigen presenting cells (APCs) during re-infection[8-10]. However, a contribution of this primary response to the generation and maintenance of memory T cell populations has not been characterized. We therefore examined longitudinal responses of endogenous antigen-specific T cells in serial adoptive transfer experiments. This allowed us to characterize the memory T cell response with precise classification of antigen experience number.

Primary response during antigen re-challenge

We used an adoptive transfer model to distinguish concurrent primary (naïve) and memory $CD8^+$ T cell responses against the same antigen upon repetitive exposure (Figure 2a).

We employed different antigen delivery systems to allow heterologous immunization and multiple challenges, while also minimizing complication from background vector[11,12]. Antigen delivery was accomplished using either plasmid DNA (pCMV-S), recombinant vaccinia virus (vHBs.4) or recombinant adenovirus (Ad-HBV).

$Ly5.1^+$ C57BL6/B10.D2 F1 mice were immunized with pCMV-S for prime and vHBs.4 for boost, and isolated spleen cells from these donor mice were used as a source of Env.28-specific memory $CD8^+$ T cells[13]. Pooled spleen cells consisted of at least 5.4% Env.28-specific memory T cells within the $CD8^+$ population, as determined by both intracellular IFN-γ and Env.28-Dimer staining (Figure 2b). Surface markers and functional properties of splenic Env.28-$Dimer^+$ $CD8^+$ T cells were $CD44^{hi}$ $CD127^{hi}$ $IL\text{-}2^+$ TNF^{++}, indicating memory phenotype (Figure 2b). Spleen cells from $Ly5.1^+$ donor mice were adoptively transferred into naïve $Ly5.2^+$ C57BL6/B10.D2 F1 recipient mice, which were subsequently treated intravenously with $5x10^9$ plaque forming units (pfu) of Ad-HBV one day after transfer[14]. Transfer of $4.1x10^7$ spleen cells (containing $5x10^5$ Env.28-$Dimer^+$ $CD8^+$ T cells) and inoculation with $5x10^9$ pfu Ad-HBV resulted in vigorous expansion of Env.28-$Dimer^+$ $CD8^+$ T cells in both the liver and spleen that peaked around day 10-14 and gradually subsided by day 35 (Figs. 2c, 2d and[15]). The majority (>90%) of Env.28-$Dimer^+$ $CD8^+$ T cells were $Ly5.1^+$; however, 5-10% were $Ly5.1^-$. No Env28-$Dimer^+$ cells that are $Ly5.1^-$ are detectable using $Ly5.1^+$ mice, which also carry Ly5.2 (data not shown). Therefore, $Ly5.1^-$ Env.28-$Dimer^+$ $CD8^+$ T cells were derived from Ly5.2 recipients (Figure 2c). For clarity, here and elsewhere we refer to this population as $Ly5.2^+$ to indicate that they originate in Ly5.2 mice. These results indicate that a primary ($Ly5.2^+$) $CD8^+$ T response is induced in response to Ad-HBV infection despite the presence of transferred $Ly5.1^+$ memory T cells (Figs. 2c and 2d). Even in heterologous immunization with recombinant viral vectors, the recall T cell response can be comprised of pre-existing memory as well as primary $CD8^+$ T cells[8-10].

Maintenance of the diversity of memory T cells

Next, to test for a primary response contribution to the overall diversity of antigen-

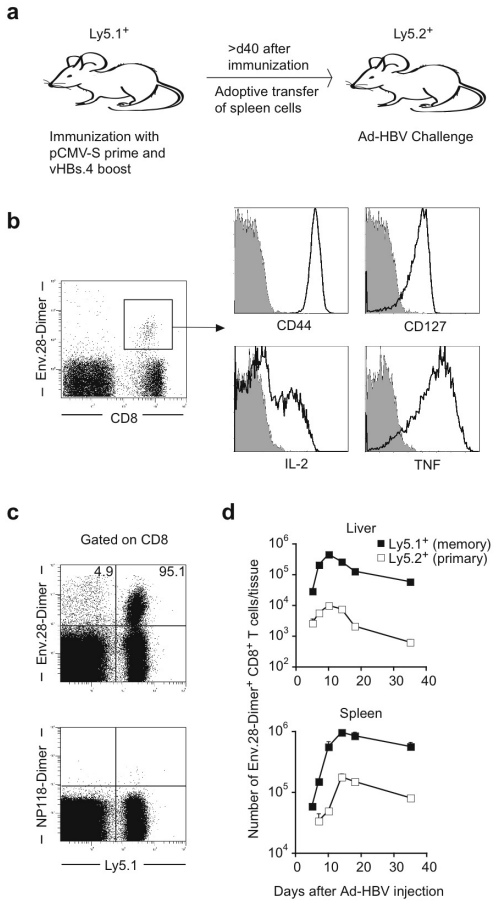

Figure. 2 A primary response during re-challenge. a, Experimental design. Ly5.1$^+$ donor mice were immunized with 100μg plasmid DNA (pCMV-S) for prime and at least four weeks later 1x10^7 pfu vHBs.4 for boost. Spleen cells (4.1x10^7 including 5x10^5 Env.28-Dimer$^+$ CD8$^+$ T cells) were transferred into naïve non-irradiated Ly5.2$^+$ mice that were then challenged one day later with 5x10^9 pfu Ad-HBV. b, Detection and phenotype of Env.28-Dimer$^+$ CD8$^+$ T cells at the time of transfer. Numbers and phenotype of Env.28-Dimer$^+$ CD8$^+$ T cells in pooled spleen cells were assessed by MHC class I Dimer staining. Filled and open histograms denote isotype control and markers, respectively. Representative data on day 85 post-vHBs.4 infection are shown. c, Env.28-Dimer$^+$ CD8$^+$ T cells in the spleen detected on day 14 post-Ad-HBV infection with 5x10^9 Ad-HBV. Numbers represent frequencies of primary (Ly5.2$^+$) and memory (Ly5.1$^+$) cells among total Env.28-Dimer$^+$ CD8$^+$ T cells. Irrelevant peptide staining (LCMV NP118) is shown as a control for Dimer staining. d, Number of Env.28-Dimer$^+$ CD8$^+$ T cells in the liver and spleen after Ad-HBV infection. The overall T cell response consists of memory (Ly5.1$^+$, filled box) and primary (Ly5.2$^+$, open box) responses. Data are indicated as mean±SEM from three to six mice analyzed per time point from three independent experiments.

specific memory T cells, we used spectratyping length analysis of complementarity determining region 3 (CDR3) with classification of the number of antigen encounters (Figure 3a). Immunized spleen cells were isolated from Ly5.1$^+$ mice on day 69 after initial challenge with pCMV-S DNA and divided into two portions. Env.28-Dimer$^+$ CD8$^+$ T cells were sorted from a half portion of spleen cells and designated as primary memory at initial challenge (population I). The remaining unsorted spleen cells (which contained 7.6x10^4 Env.28-specific CD8$^+$ T cells) were then transferred into naïve Ly5.2$^+$ mice. Recipient mice were challenged with 1x10^7 pfu vHBs.4 as a re-challenge and sacrificed on day 8 post-infection. Splenic Ly5.1$^+$ or Ly5.1$^-$ Env.28-Dimer$^+$ CD8$^+$ T cells were sorted separately and designated as secondary effector at re-challenge (population II) and primary effector at re-challenge (population III), respectively (Figure 3a). Total RNAs from these CD8$^+$ T cells were examined for TCR transcript diversity by spectratyping analysis[16].

Analysis of Env.28-Dimer$^+$ CD8$^+$ T cell populations revealed that primary memory T cells (I) have a TCR repertoire that is skewed compared to naïve CD8$^+$ spleen cells in each Vβ gene usage (Figure 3b). The TCR repertoire of secondary effector T cells (II) was further narrowly focused among the majority of Vβ genes used, indicating selective expansion of pre-existing memory cell subpopulations in the recall response (Figure 3b, Vβ8.1 and 8.3). Compared with secondary effector (II), primary effector at re-challenge (III) consisted of a broader TCR repertoire, with profile peaks that differed from those of secondary effector (Figure 3b, Vβ2, 8.1 and 8.3). Alternative patterns were detectable in some Vβ genes, indicating that this primary response to re-challenge recovered Vβ usage that was undetectable in the pre-existing memory T cell population (Figure 3b, Vβ3.1, compare III to I and II). Since populations II and III were concurrently generated in

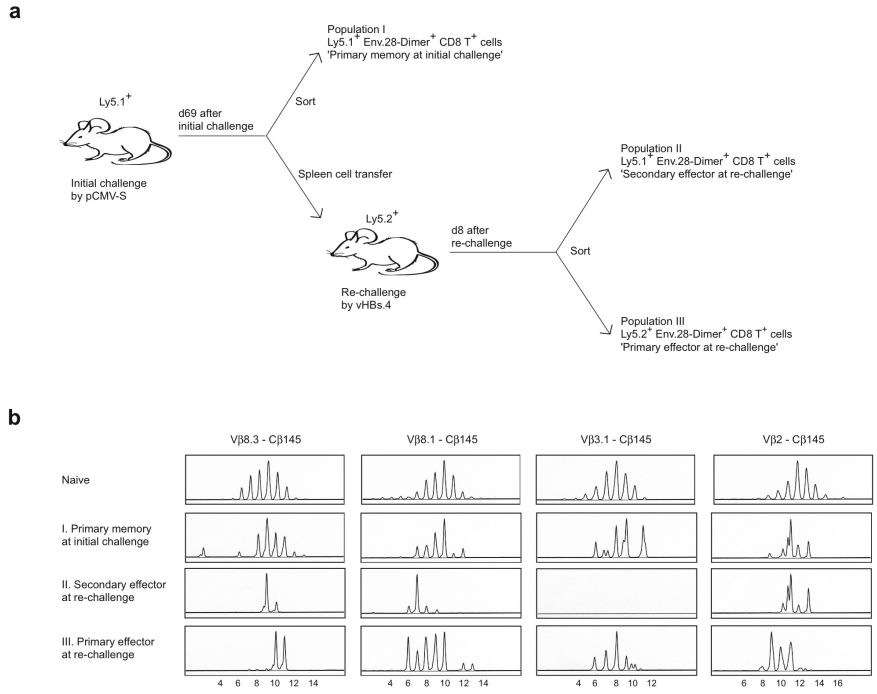

Figure 3 TCR repertoire analysis of antigen-specific $CD8^+$ T cells after recall.
a, Experimental design. Env.28-$Dimer^+$ $CD8^+$ T cells (primary memory at initial challenge, population I) were purified by flow cytometry from a half portion of $Ly5.1^+$ spleen cells on day 69 after immunization intramuscularly with pCMV-S (initial challenge). The remainder of spleen cells (containing $7.6x10^4$ Env.28-$Dimer^+$ $CD8^+$ T cells) were transferred into naïve non-irradiated $Ly5.2^+$ mice. Recipient mice were subsequently challenged intravenously with $1x10^7$ pfu vHBs.4 (secondary challenge) and sacrificed on day 8 post-infection. $Ly5.1^+$ or $Ly5.1^-$ Env.28-$Dimer^+$ $CD8^+$ T cells (secondary effector at re-challenge, population II, and primary effector at re-challenge, population III, respectively) were sorted separately from spleen cells. The same number (at least $3x10^4$) of naïve $CD8^+$ T cells were also analyzed as a control. Purity of all isolated samples was at least 97% (data not shown). All procedures were carried out using individual mice. b, Profiles of the Vβ8.3, Vβ8.1, Vβ3.1 and Vβ2 subrepertoires in spleen cells from naïve (top), primary memory at initial challenge (population I, upper middle), secondary effector at re-challenge (population II, lower middle), and primary effector at re-challenge (population III, bottom). x-axis, length in amino acids of the CDR3 regions; y-axis, fluorescence intensity, reflecting the number of clones using each Vβ/CDR3 length combination. The four panel sets within Vβ subrepertoires are from an individual mouse. Results are representative of three independent experiments (n=3-4 per experiment).

individual mice, they can be considered together as an indication of the overall diversity of the recall response. The TCR repertoire diversity of the overall secondary response (II+III) was therefore restored to more closely approximate that in the initial primary response (I). Our analysis revealed that the narrowly skewed TCR repertoire of secondary effector (II) is complemented by the broader TCR repertoire of primary effector at re-challenge (III) in more than 75% of Vβ genes.

Maintenance of the proliferative capacity of memory T cell population

We next investigated the functional difference of primary and secondary memory populations in response to further antigen challenge by monitoring their proliferation upon tertiary challenge in vivo (Figure 4a).

Using serial transfer experiments, we analyzed antigen-specific T cell responses in the same animal upon repetitive challenge with precise classification of antigen encounter

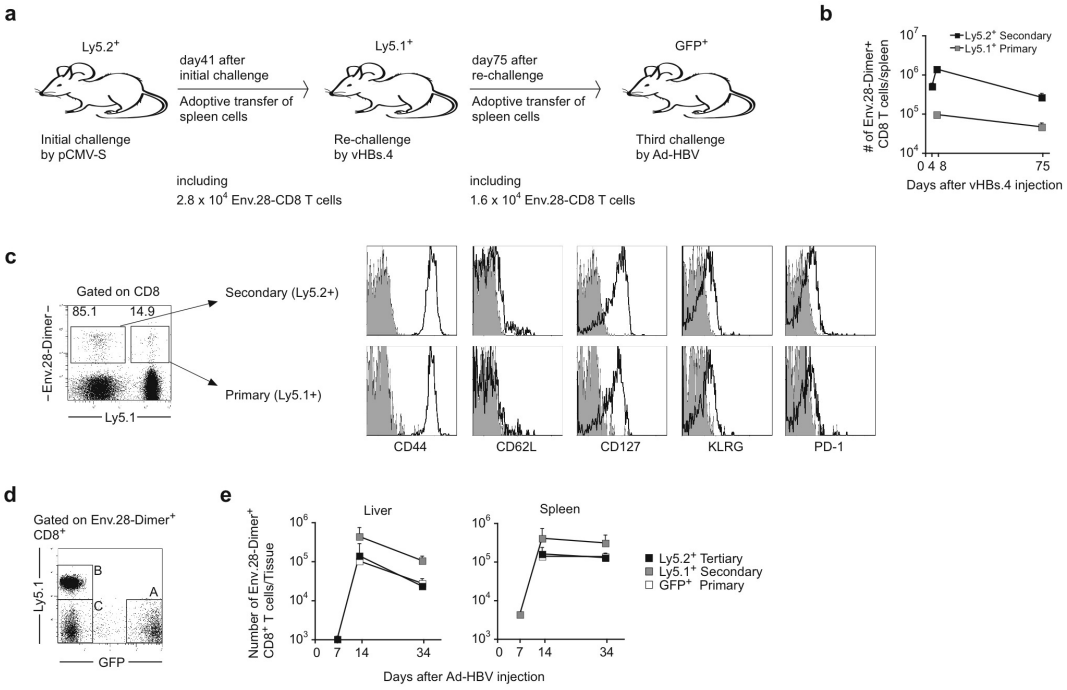

Figure 4 In vivo proliferation of primary (new) and secondary (old) memory T cells. a, Experimental design. Spleen cells containing Env.28-Dimer$^+$ CD8$^+$ T cells from Ly5.2$^+$ mice immunized intramuscularly by plasmid DNA (initial challenge) were adoptively transferred into naïve non-irradiated Ly5.1$^+$ mice. Ly5.1$^+$ mice were then challenged intravenously with 1x10^7 pfu vHBs.4 (re-challenge), and 75 days later spleen cells containing both Ly5.2$^+$ secondary memory and Ly5.1$^+$ primary memory cells were further adoptively transferred into naïve GFP$^+$ mice. Env.28-Dimer$^+$ CD8$^+$ T cell populations were analyzed after challenging GFP$^+$ mice with Ad-HBV (third challenge). b, c, Transferred spleen cells contain both secondary memory and primary memory after re-challenge. (b) Kinetics of secondary (Ly5.2$^+$) and primary (Ly5.1$^+$) Env.28-Dimer$^+$ CD8$^+$ T cells (filled and dotted boxes, respectively) in the spleen of Ly5.1$^+$ mice and (c) flow cytometry profiles gated on CD8$^+$ T cells of Ly5.1$^+$ spleen cells on day 75, after re-challenge (vHBs.4). At the time of adoptive transfer, 5.6x10^7 transferred spleen cells included 1.6x10^5 Env.28-Dimer$^+$ CD8$^+$ T cells, which consisted of more Env.28-Dimer$^+$ secondary memory cells that were Ly5.2$^+$ than Ly5.1$^+$ primary (85.1% and 14.9%, respectively). d, Memory T cells generated in a more recent challenge form the largest effector population in a subsequent challenge. Dot plots gated on Env.28-Dimer$^+$ CD8$^+$ T cells show the distribution by origin of antigen-specific CD8$^+$ T cells in the liver on day 14 post-Ad-HBV infection. A, primary effector (GFP$^+$); B, secondary effector (Ly5.1$^+$); C, tertiary effector (Ly5.2$^+$). e, Number of tertiary (filled box), secondary (dotted box) and primary (open box) CD8$^+$ T cells that were Env.28-Dimer$^+$ in the liver and spleen at various time-points after Ad-HBV injection.

number. Spleen cells from Ly5.2$^+$ mice immunized with pCMV-S (initial challenge) were adoptively transferred into Ly5.1$^+$ mice, which were subsequently infected with 1x10^7 pfu vHBs.4 (re-challenge). Seventy-five days post-infection, spleen cells from these Ly5.1$^+$ recipient mice (containing 1.6x10^5 Env.28-Dimer$^+$ CD8$^+$ T cells, and consisting of 85.1 % Ly5.2$^+$ secondary memory and 14.9% Ly5.1$^+$ primary memory cells) were transferred into naïve GFP$^+$ mice (Figs. 4b, c). At the time of transfer, both primary and secondary memory cells exhibited comparable surface and functional phenotypes (Figure 4c). The GFP$^+$ recipient mice were then challenged with 5x10^9 pfu Ad-HBV (third challenge), and the subsequent T cell response was analyzed.

At the peak of memory CD8$^+$ T cell response after Ad-HBV injection (day 14), Env.28-Dimer$^+$ CD8$^+$ T cells that were GFP$^+$Ly5.1$^-$, GFP$^-$ Ly5.1$^+$, and GFP$^-$Ly5.1$^-$all were detectable. This indicates that the memory T cell pool to the same antigen specificity upon tertiary challenge is a mixture of primary (GFP$^+$), secondary

(Ly5.1$^+$), and tertiary (GFP$^-$Ly5.1$^-$) cells (Figure 4d). Surprisingly, 14 days after tertiary challenge, effector cells derived from secondary challenge (GFP$^-$Ly5.1$^+$) outnumbered those derived from the initial primary challenge (GFP$^-$Ly5.2$^+$) and formed the majority of the memory CD8$^+$ T cell pool (tertiary : secondary : primary = 22.6% : 57.7% : 19.7% ; Figs 4d, e). These data indicate that the newly formed primary memory cell population has more expansion potential - i.e. proliferative and/or survival potential - than the older memory cell population upon subsequent antigen challenge. Consistent with our previous observations, primary (GFP$^+$Ly5.1$^-$) Env.28-Dimer$^+$ CD8$^+$ T cells were also observed after third challenge (Figure 4e, white box), indicating that a primary response potentially occurs upon every recall response.

Primary response rejuvenates memory T cell population

This study provides new insights into the generation and maintenance of memory T cell populations. We have demonstrated that a primary CD8$^+$ T cell response during antigen re-challenge can qualitatively and quantitatively contribute to the TCR diversity and proliferative potential of the antigen-specific memory T cell population. It has been suggested that memory T cells might resemble stem cells in their longevity and self-renewal capacity, which allows for the continual generation of descendent effector cells[6,7,17]. In the memory maintenance phase, memory T cells self-renew through homeostatic proliferation in the presence of cytokines such as interleukin (IL)-15, IL-2 and IL-7, but independent of antigen and major histocompatibility complex (MHC) [6,18]. Furthermore, a recent study identified CD44^{lo}CD62L^{hi}CD8$^+$ T cells as candidate memory stem cells with the capacity to generate central memory, effector memory and effector subsets while self-renewing in a graft-versus-host disease (GVHD) model[19]. However, our results indicate that re-exposure to the same antigenic determinant causes the TCR repertoire of memory T cells to skew significantly, with newly generated clones having CDR3 length profiles that differ from those of pre-existing memory T cells. As a result, when thymic output continuously reconstitutes peripheral T cells, overall TCR diversity is maintained through the inclusion of newly formed primary T cells in the memory T cell pool (Figure 3b). Given that even a small gain in TCR repertoire diversity to a single viral epitope can result in higher resistance to pathogen[20], this may afford greater versatility in controlling future infections. In addition, our serial transfer experiments indicate that this new memory T cell population expands more vigorously than older ones, and is therefore a major memory cell responder and effector in future challenges (Figs. 4d, e). Consistent with previous studies using TCR Tg T cells exposed to repetitive challenge, this suggest that each memory cell itself is not immortal[21,22]. Taken together, these results suggest that in acute infections, repetitive 'explosive' proliferation results in bias against established memory T cells, resulting in a memory T cell population that is dynamically turned over in favor of new conscripts (Figure 5).

Therefore, replenishing the supply of primary T cells in the memory pool upon each recall response may be important for maintaining optimal memory T cell response and longevity.

Conclusion

A major goal of vaccination is to generate long-lived protective memory T cells. The precise mechanisms that control T cell expansion and lifespan during repetitive infection by complex pathogens is not well understood. It is further unclear whether newly formed memory T cells have better protection capacity than pre-existing memory cells. Nonetheless, our data suggests that the ideal prime-boost strategy in vaccination

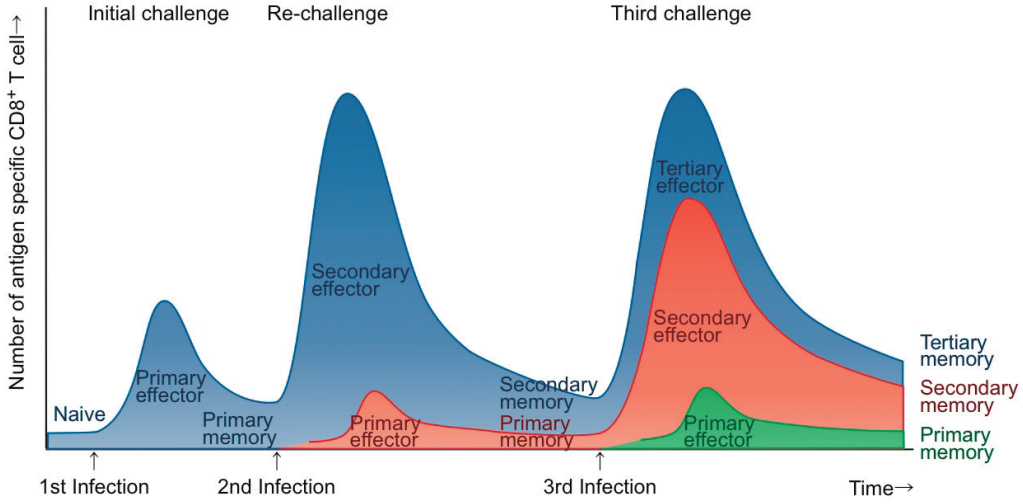

Figure 5 Schematic representation of turnover in the antigen-specific memory $CD8^+$ T cell population upon repetitive infection. Primary memory T cells generated in the first infection (blue) expand vigorously and form the majority of effector T cells upon a second infection. Some primary effector T cells (red) are also newly generated, and the antigen-specific memory T population after clearance is comprised of both primary memory (red) and secondary memory (blue). On subsequent infection, the majority of antigen-specific T cells arise from memory T cells generated in most recent challenge (red). In addition, yet another primary response occurs (green). In this model, memory $CD8^+$ T cell populations are heterogeneous in antigen encounter number, and dynamically turn over upon repetitive challenge.

should be directed to balance the expansion of pre-existing memory T cells with the induction of primary T cells for optimal memory pool size, repertoire diversity and proliferative potential.

The currently accepted classifications of memory T cell subpopulations are as central memory and effector memory, distinguished mainly by location and time after antigen clearance[23]. Our results introduce another perspective on the heterogeneous feature of memory T cells, one that is classified according to the number of antigen encounters.

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