Maintenance of memory CD8$^+$ T cell diversity and proliferative potential by a primary response upon re-challenge

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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 show that a primary response during re-challenge significantly contributes to memory T cell pool both qualitatively and quantitatively. Upon re-challenge, the skewed V$\beta$ usage and TCR repertoire of pre-existing memory T cells is partly corrected by diversity in a newly primed (primary) T cell population. Importantly, this primary population expands more vigorously in a subsequent antigen encounter. 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.

Introduction

The memory T cell response ensures that a recall response to foreign antigen is greater in magnitude and faster than a naive T cell response. This enhanced response results in part through the increased frequency, proliferative responsiveness and effector function acquisition of memory T cells compared with naive T cells (1–3). 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 (2, 4–9). 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 (2).

Although a recall T cell response consists mostly of pre-existing (secondary) CD8$^+$ T cells, some naive (primary) CD8$^+$ T cells are newly primed by antigen-presenting cells (APCs) during re-infection (10–12). This was shown in studies that employed either TCR transgenic (Tg) or non-TCR Tg systems. However, the role of this primary response in the maintenance of memory T cell populations has not been thoroughly characterized. Furthermore, the endogenous polyclonal T cell response presumably follows a different developmental program than TCR Tg cells, possibly due to the different number of cell divisions and/or differential competition for APCs and co-stimulatory/growth signals in these systems (13, 14). Memory T cells are classified as CD62L$^+$CCR7$^+$ effector memory T cells (T$\text{EM}$) or CD62L$^-$CCR7$^+$ central memory T cells (T$\text{CM}$) based on homing characteristics and effector function (2, 15). While studies using large numbers of monoclonal TCR Tg T cells indicate a lineage relationship between T$\text{EM}$ and T$\text{CM}$ (13), antigen-specific T cells generated from low numbers of endogenous naive precursors (and which experience a higher number of cell divisions) remain T$\text{EM}$ and fail to convert to T$\text{CM}$ (14). Taken together, these results indicate that initial precursor frequency is important in the study of in vivo immune responses. 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. Our analysis has the advantage of more closely approximating the natural dynamics of memory T cell populations in response to serial antigen challenge.

Consistent with previous reports, we find that antigen re-challenge can elicit a primary response despite the presence of pre-existing memory T cells. We further find that this primary response contributes to overall TCR diversity within the antigen-specific memory T cell population. Surprisingly,
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effector T cells generated from a more recent challenge expand more robustly in a third antigen encounter. These results indicate that a primary response during antigen re-exposure can contribute to the maintenance of memory T cell populations, and that these populations may ultimately be subject to dynamic turnover.

Methods

Mice

B10.D2 and C57BL/6 were purchased from SLC (Shizuoka, Japan). C57BL/6 background GFP and Ly5.1+ mice were purchased from RIKEN (Japan) and The Jackson Laboratory (Bar Harbor, ME, USA), respectively. C57BL/6 GFP+/B10.D2 F1 (H-bxd), C57BL/6 Ly5.2/B10.D2 F1 and C57BL/6 Ly5.1/B10.D2 F1 mice were generated by crossing C57BL/6 background GFP+ with B10.D2, C57BL/6 with B10.D2 and Ly5.1+ with B10.D2, respectively. Mice were aged 6–12 weeks at experimental onset and maintained by in-house breeding at the University of Tokyo under specific pathogen-free conditions and in accordance with the University of Tokyo Institutional Animal Care and Use Committee guidelines.

Peptide, plasmid and recombinant vaccinia virus and adenovirus

A peptide corresponding to a known H-2 Ld-restricted HBs antigen-specific CD8+ T cell epitope (S28–39; IPQSLDSWTS) and lymphocytic choriomeningitis virus NP118 (RPOASGVYM) were synthesized by Sigma Genosys (Ishikari, Japan) (16). A plasmid that expresses HBs antigen under transcriptional control of the CMV immediate early promoter (pCMV-S) was kindly provided by Aldevron (Fargo, ND, USA) (17). Recombinant vaccinia virus encoding the small Env protein of hepatitis B virus (HBV) (vHBs.4) was kindly provided by Moss (NIAID) (18, 19). A replication-defective adenovirus vector containing a 1.3-fold-overlength replication competent HBV genome that drives liver-specific HBV antigen expression (Ad-HBV) was kindly provided by Protzer (University of Heidelberg, Heidelberg, Germany) (20).

Immunization of mice and adoptive transfer

In prime-boost experiments, mice were immunized once with pCMV-S injected into regenerating tibialis anterior muscles (50 μg per leg) 5 days after the injection of cardiotoxin (LATOXAN, Valence, France). At least 4 weeks after DNA injection, mice were intravenously administered a booster injection of 1 × 10^7 plaque-forming units (pfu) of vHBs.4 (21). Mice were sacrificed at least 40 days after and isolated spleen cells were injected intravenously into congenic non-irradiated naive recipient mice after treatment with ACK lysis buffer (0.15 M NH4Cl, 10 mM KHCO3 and 0.1 mM Na2EDTA at pH 7.4). Recipient mice were infected intravenously 1 day later with Ad-HBV (5 × 10^8 pfu), as described (20). For ovaly vHBs.4 titration, plaque assay of homogenized ovaries were performed by 10-fold serial dilutions. Titers were determined by staining with 0.1% crystal violet after 48 h (13).

Lymphomononuclear cell preparation

Spleen cells were isolated by passage through a 70-μm cell strainer (Becton Dickinson) with a 2.5-ml syringe plunger and washed twice with PBS. For intrahepatic lymphocyte (IHL) isolation, livers were perfused with 10 ml of PBS via portal vein to remove circulating lymphocytes and liver cell suspensions were pressed through 200-μm stainless mesh. IHLs were isolated by 33% Percoll (Amersham Bioscience, Piscataway, NJ, USA) centrifugation of homogenized liver for 10 min at 800 × g and washed twice with RPMI 1640 medium for further use. For separation of secondary memory cells in Fig. 5, magnetic microbeads and AutoMACS (Miltenyi Biotec, Germany) were used according to manufacturer's protocol.

FACS analysis

All antibodies used in flow cytometry were purchased from BD Pharmingen (San Diego, CA, USA), except for CD127 (eBioscience; San Diego, CA, USA), Dimer X (BD Pharmingen), which consists of recombinant H-2 Ld-Ig fusion protein supplemented with recombinant β2 microglobulin, was conjugated to Env.28-Dimer (Env.28-Dimer) according to the supplied protocol. Single-cell suspensions of lymphocytes were prepared and 10^6 cells were incubated in PBS containing Env.28-Dimer and antibody supplemented with 2% fetal bovine serum for 1 h at 4°C. After washing twice with PBS, cells were stained with PE-conjugated anti-mouse IgG1 at 4°C for 30 min to detect Env.28-Dimer+ cells. Intracellular cytokine staining for IFN-γ was performed using Cytofix/Cytoperm (BD Pharmingen). Briefly, lymphocytes were cultured in the presence or absence of Env.28-39 peptide (0.2 μg ml−1) at 37°C in complete medium supplemented with 50 U ml−1 human recombinant IL-2 and brefeldin A for 5 h (IFN-γ and tumor necrosis factor (TNF)) or 6 h (IL-2), followed by surface staining for CD8 and Ly5.1 and intracellular staining for IFN-γ, TNF, and IL-2. Flow cytometry was performed using an EPICS, ELITE and ALTRA (Beckman Coulter, Fullerton, CA, USA), and data were analyzed with Expo32 software (Beckman Coulter).

TCR repertoire analysis

Spectratyping analyses were performed as described (22). Briefly, total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) from sorted Env.28-Dimer+ CD8+ T cells. cDNAs were synthesized using a SuperScript III cDNA synthesis kit (Invitrogen) with oligo dT primer. Different sets of 24 PCRs were performed with CACTGATGTTCTGTGT-145 (CACTGATGTTCTGTGT-GACA) and 24 unique Vβ-specific primers (22). Following amplification, five cycle run-off reactions were performed using fluorescent primer Cβ 5’ (CTTGGGTGGAGTCACATTCTC). PCR products were analyzed using an ABI3730 sequencer (Applied Biosystems, Foster City, CA, USA), and data were analyzed with Gene Mapper Software version 3.1 (Applied Biosystems). For TCR repertoire analysis, all experimental procedures including adoptive transfer of spleen cells and sorting of Env.28-Dimer+ CD8+ T cells were carried out on individual mice.

Statistical analysis

The two-sample t-test was used to evaluate the correlation between data. Statistical analyses were performed with the JMP software, version 6.0.0 (SAS Institute Inc., Cary, NC, USA).
Results

Primary (naive) CD8+ T cells can be generated during a recall response

We used an adoptive transfer model to distinguish concurrent primary (naive) and memory CD8+ T cell responses against the same antigen upon repetitive exposure (Fig. 1A). We chose as an antigen the HBV surface antigen that includes the Env.28 peptide (S28–39), an immunodominant, Ld-restricted CD8+ T cell epitope located between residues 28 and 39 (16). In addition, we employed different antigen delivery systems to allow heterologous immunization and multiple challenges, while also minimizing complication from background vector (23, 24). Antigen delivery was accomplished using plasmid DNA (pCMV-S), recombinant vaccinia virus (vHBs.4) or recombinant adenovirus (Ad-HBV). Details are provided in Methods.

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 (21). 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 (Fig. 1B and Supplementary Figure 1, available at International Immunology Online). Surface markers and functional properties of splenic Env.28-Dimer+ CD8+ T cells were CD44hi CD127hi IL-2+ TNF++, indicating memory phenotype (Fig. 1B and Supplementary Figure 1, available at International Immunology Online). Spleen cells from Ly5.1+ donor mice were adoptively transferred into naive Ly5.2+ C57BL6/B10.D2 F1 recipient mice, which were subsequently treated intravenously with 5 × 10^6 pfu of Ad-HBV 1 day after transfer (20). Transfer of 4.1 × 10^7 spleen cells (containing 5 × 10^5 Env.28-Dimer+ CD8+ T cells) and inoculation with 5 × 10^9 pfu Ad-HBV resulted in vigorous expansion of Env.28-Dimer+ CD8+ T cells in both the liver and spleen that peaked around days 10–14 and gradually subsided by day 35 (Fig. 1C and D and (25)). The majority (>90%) of Env.28-Dimer+ CD8+ T cells were Ly5.1+, however, 5–10% were Ly5.1-. No Env28-Dimer+ T cells are Ly5.1+ but 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 (Fig. 1C). 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 (Fig. 1C and D). 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 (10–12). A primary response occurs with quantitative expansion of pre-existing memory cells

Next, to examine factors that might influence the induction of a primary response, we transferred different numbers of Ly5.1+–immunized spleen cells (4.1 × 10^5 or 4.1 × 10^5), containing 5 × 10^6 or 5 × 10^7 Env.28-specific CD8+ T cells, into naive Ly5.2+ mice (Fig. 2). Recipient mice were challenged 1 day later with different doses of Ad-HBV (5 × 10^9 or 1 × 10^9 pfu). Although a rapid and vigorous memory CD8+ T cell response was induced in all groups, primary (Ly5.2+) Env.28-Dimer+ CD8+ T cells were only observed in
mice infected with $5 \times 10^8$ pfu of Ad-HBV (Fig. 2A, Groups 1 and 2). This suggests that antigen dose can determine whether a primary response detectably occurs. Furthermore, the magnitude of the primary response was variable, with ~25-fold higher induction when fewer ($5 \times 10^4$) memory cells were transferred (Fig. 2A).

We next tested whether a correlation exists between pre-existing memory cell division and the induction of a primary response (Fig. 2B). We labeled Ly5.1$^+$-immunized spleen cells with 5,6-carboxyfluoroscein succinimidyl ester before transfer of $5 \times 10^5$ memory cells and challenge with different doses of Ad-HBV ($5 \times 10^9$ or $1 \times 10^8$ pfu; Groups 2 and 3, respectively, Fig. 2A and B). Nearly all transferred memory CD8$^+$ T cells had divided by day 14 in mice injected with a high Ad-HBV dose, in which a primary CD8$^+$ T cell response occurred (Fig. 2A and B, Ly5.1$^+$). In contrast, 10–20% of transferred memory CD8$^+$ T cells were undivided over 35 days in mice receiving a low Ad-HBV dose, in which a primary response was undetectable (Fig. 2A and B, Ly5.2$^+$). This suggests that a primary response is induced upon recall only under conditions where antigen load saturates pre-existing memory CD8$^+$ T cell expansion, when presumably excess antigen is present and available to less efficient naive T cells. Consistent with previous observations, once recruited into division, memory T cells underwent at least 7–8 divisions (26). Furthermore, in mice infected with $1 \times 10^6$ pfu Ad-HBV, the undivided population was stably maintained after day 7, whereas the number of divided cells had peaked by this time.

The primary response contributes to overall TCR diversity
To investigate the primary response contribution to overall TCR diversity, we used V$\beta$ usage analysis and spectratyping length analysis (immunoscope) of complementarity determining region 3 (CDR3), with classification of the number of antigen experiences. As CDR3 segments are the most polymorphic regions of TCR chains, their diversity reflects T cell population complexity (27). Immunized spleen cells were isolated from Ly5.1$^+$ mice on day 69 after initial challenge with pCMV-S and divided into two portions. Env.28-Dimer$^+$ CD8$^+$ T cells were sorted from half portion of spleen cells (primary memory from initial challenge) and designated as population I. The remaining unsorted spleen cells (which contained $7.6 \times 10^4$ Env.28-Dimer$^+$ CD8$^+$ T cells) were then transferred into naive Ly5.2$^+$ mice. Recipient mice were challenged with $1 \times 10^7$ pfu vHBs.4 as a re-challenge and sacrificed on day 8 after infection. Splenic Ly5.1$^+$ or Ly5.1$^+$ Env.28-Dimer$^+$ CD8$^+$ T cells were sorted separately and designated as secondary effector from re-challenge (population II) and primary effector from re-challenge (population III), respectively (Fig. 3A). V$\beta$ usage of these populations was analyzed by flow cytometry (Fig. 3B). In addition, total RNAs from these CD8$^+$ T cells were examined for TCR transcript diversity by spectratyping analysis (22).
Compared with naive CD8+ T cells, population I predominantly used V8.2, 4 and 14, although other V8 usage was detectable (Fig. 3B). After re-challenge, the distribution of this population dramatically skewed, with V8.2, 4 and 10 comprising 75% of V8 gene usage in population II. Population III, which concurrently existed in Ly5.2+ recipients, displayed a wide range of V8 gene usage, including V8.5.1/5.2, 6, 7, 8.1/8.2, 8.3 and 11.

Spectratyping length analysis of Env.28-Dimer+ CD8+ T cell populations revealed that population I has a TCR repertoire that is skewed compared with naive CD8+ T cells in each V8 usage (Fig. 3C). The TCR repertoire of population II was further narrowly focused among the majority of V8 genes tested, indicating selective expansion of pre-existing memory cell sub-populations in the recall response (Fig. 3C, V8.4, 5.1, 6, 7, 8.1, 8.2, 8.3, 9 and 16). However, population III consisted of a broader TCR repertoire when compared with population II. With some V8 genes, a skew in secondary effector was accompanied by different profiles in primary effector (Fig. 3C, V8.4, 6, 7, 8.2, 8.3, 9 and 16). In others, multiple primary effector clones were generated, some of which overlapped with secondary effector (Fig. 3C, V8.B1 and 13). This may indicate that primary effector can compete with identical CDR3 length clones in the established memory population. In still other cases, primary effector recovered V8 usage that was absent and/or undetectable in the pre-existing secondary effector population (Fig. 3B and C, V8.B3.1), and could even contribute to overall diversity in the absence of dramatic skewing (Fig. 3C, V8.2 and 11). Since populations II and III were concurrently generated in individual mice, they can be considered together as the cumulative recall response. The overall diversity of this response was therefore restored to more closely approximate that from the initial challenge (Fig. 3B and C). The narrowly skewed TCR repertoire of secondary effector was corrected, to some extent, by diversity in the primary effector population in >75% of V8 genes analyzed (Fig. 3C).

Newly emerged primary memory cells dominate in future recall responses

We next tested for a functional difference between primary and secondary memory populations by monitoring their response to a third antigen challenge (Fig. 4A). Spleen cells from Ly5.2+ mice immunized with pCMV-S (initial challenge) were adoptively transferred into Ly5.1+ mice, which were subsequently infected with 1 × 107 pfu vHBs.4 (re-challenge). Seventy-five days after infection, spleen cells from Ly5.1+ recipient mice (containing 1.6 × 106 Env.28-Dimer+ CD8+ T cells, comprised of 85.1% Ly5.2+ secondary memory and 14.9% Ly5.1+ primary memory cells) were transferred into naive GFP+ mice (Fig. 4B and C). At the time of transfer (day 75 after re-challenge with vHBs.4), primary and secondary memory cells exhibited comparable phenotypes (CD44hi CD62Llo, Fig. 4C and Supplementary Figure 2, available at International Immunology Online) and ability to produce IFN-γ upon stimulation with titrated doses of S28–39 peptides (Fig. 4D). Primary and secondary memory T cells achieved 50% maximal IFN-γ production at 2.3 × 108 M and 4.3 × 1018 M of S28–39 peptides, respectively. This suggested that the functional avidities of both memory populations were similar. GFP+ recipient mice were then challenged with 5 × 109 pfu Ad-HBV (third challenge), and subsequent T cell responses were analyzed.

At the peak of memory CD8+ T cell response after Ad-HBV injection (day 14), Env.28-Dimer+ CD8+ T cells were detectable that were GFP+Ly5.1+, GFP+Ly5.1+ or GFP+Ly5.1+ (Fig. 4E). This indicates that the memory T cell pool to the same antigen specificity upon a third challenge is a mixture of primary (GFP++), secondary (Ly5.1+) and tertiary (Ly5.2+) cells. Surprisingly, 14 days after Ad-HBV challenge, effector cells derived from re-challenge (Ly5.1+) outnumbered those from the initial challenge (Ly5.2+) and formed the majority of the memory CD8+ T cell pool (primary:secondary:tertiary = 19.7%:57.7%:22.6%; Fig. 4E and F). To eliminate biased cell survival of Ly5.1+ and Ly5.2+ cells as a potential explanation, we confirmed these results in a reversal of congenic backgrounds (Ly5.1+ → Ly5.2+ → GFP, Supplementary Figure 3, available at International Immunology Online). Furthermore, as GFP+ Env.28-Dimer+ CD8+ T cells were observed after a third challenge (Fig. 4F and Supplementary Figure 3D, open box, available at International Immunology Online), a primary response potentially occurs upon every recall response.

Primary memory cells have better proliferative capacity and similar anti-viral activity compared with secondary memory cells

While these experiments approximate in vivo conditions, where different memory populations co-exist in the same animal, we also examined the properties of these populations when transferred independently of one another. Equal numbers of these cell populations (1.4 × 107) were adoptively transferred together with naive spleen cells (1 × 106) into recipient mice (Fig. 5A). To obtain a sufficient number of primary memory cells, primary and secondary memory cells were obtained from separate mice. Primary memory cells were transferred from Ly5.1+ mice on day 74 after pCMV-S immunization. For secondary memory cell transfer, Ly5.1+ mice were initially challenged with pCMV-S. Sixty-eight days later, 2.7 × 107 spleen cells containing 2.7 × 106 primary memory cells were adoptively transferred into Ly5.2+ mice, which were then sacrificed on day 74 after Ad-HBV infection. Ly5.1+ secondary memory cells were selectively isolated from spleen using magnetic microbeads to eliminate Ly5.2+ primary memory cells. Mice that received 1 × 109 Ly5.1+ naive spleen cells were also analyzed. One day after transfer, recipient mice were challenged with 5 × 109 pfu vHBs.4, and the proliferation and anti-viral properties of primary and secondary memory cells were analyzed on day 5 after vHBs.4 infection.

In primary memory recipients (Group 1), Env.28-Dimer+ CD8+ T cells expanded vigorously and comprised 28.9% of Ly5.1+ donor-derived CD8+ T cells after vHBs.4 infection [Group 1, Fig. 5B (upper right panel) and C]. In secondary memory recipients (Group 2), this frequency was 5.0% [Group 2, Fig. 5B (middle right panel) and C]. While this represents a significant expansion when compared with initial frequency (~1.6% of Ly5.1+ donor-derived CD8+ T cells), it is less than that observed in the primary memory population. Indeed, the number of Ly5.1+ Env.28-Dimer+ CD8+ T cells was 5.2 × 106 and 2.7 × 105 in the spleen of primary memory recipients (Group 1) and secondary memory recipients (Group 2), respectively—a difference of nearly 20-fold.
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A

Population I
Ly5.1+ Env28-Dimer+ CD8 T cells
‘Primary memory from initial challenge’

Initial challenge with pCMV-S

Sort

d8 after initial challenge

Spleen cell transfer

Ly5.2+

Population II
Ly5.1+ Env28-Dimer+ CD8 T cells
‘Secondary effector from re-challenge’

Population III
Ly5.2+ Env28-Dimer+ CD8 T cells
‘Primary effector from re-challenge’

Re-challenge with vHBs.4

B

Percent of Vβ usage within each memory populations

C

Vβ1 - CJ145
Vβ2 - CJ145
Vβ3.1 - CJ145
Vβ4 - CJ145
Vβ5.1 - CJ145
Vβ6 - CJ145

Vβ7 - CJ145
Vβ8.1 - CJ145
Vβ8.2 - CJ145
Vβ8.3 - CJ145
Vβ9 - CJ145
Vβ10 - CJ145

Vβ11 - CJ145
Vβ13 - CJ145
Vβ16 - CJ145

Naive
Population I
Population II
Population III

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(P < 0.0001, Fig. 5C). Primary memory cells in naive cell recipients (Group 3) were barely detectable [Group 3, Fig. 5B (lower right panel) and C]. We also examined the anti-viral capacities of these cell populations (Fig. 5D). Compared with naive mice (Group 3), mice receiving memory cells—either primary or secondary—had a reduction in viral load of ~20-fold in their ovaries (P < 0.0001). Therefore, despite differences in proliferative capacity, both memory cell populations offered comparable protection under these experimental conditions. Nonetheless, these

Fig. 4. Primary and secondary memory T cell dynamics in a third antigen challenge. (A) Experimental design. Spleen cells from Ly5.2+ mice immunized with pCMV-S (initial challenge) were adoptively transferred into naive non-irradiated Ly5.1+ mice. Ly5.1+ mice were then challenged intravenously with 1 × 10^7 pfu vHBs4 (re-challenge), and 75 days later, spleen cells containing both Ly5.2+ secondary and Ly5.1+ primary memory cells were adoptively transferred into naive GFP+ mice. Env.28-Dimer+ CD8+ T cell populations were analyzed after a third challenge with 5 × 10^5 pfu Ad-HBV (third 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. (C) Flow cytometry of spleen cells from Ly5.1+ mice 75 days after re-challenge with vHBs4 (left). At the time of adoptive transfer, 5.6 × 10^7 transferred spleen cells included 1.6 × 10^5 Env.28-Dimer+ CD8+ T cells, consisting of more Ly5.2+ than Ly5.1+ memory cells (85.1 and 14.9%, respectively). Right, phenotype of Env.28-Dimer+ CD8+ T cells at the time of transfer. Filled and open histograms denote isotype control and markers, respectively. (D) IFN-γ production by primary and secondary memory cells was examined after 5 h of stimulation with indicated doses of S28–39 peptides. The results are expressed as a percentage of the maximum response attained with saturating peptide concentrations (10^-6 M). (E) Dot plots showing the distribution by origin of Env.28-Dimer+ CD8+ T cells in the liver of GFP+ mice on day 14 after Ad-HBV infection. A, primary effector (GFP+); B, secondary effector (Ly5.1+) and C, tertiary effector (Ly5.2+). (F) Number of tertiary (filled box), secondary (dotted box) and primary (open box) Env.28-Dimer+ CD8+ T cells in the liver and spleen of GFP+ mice at various time points after Ad-HBV injection. Data are indicated as mean ± SEM of three to five mice per group.

Fig. 3. TCR repertoire analysis of antigen-specific CD8+ T cells after re-challenge. (A) Experimental design. Env.28-Dimer+ CD8+ T cells were sorted from a half portion of Ly5.1+ spleen cells on day 69 after challenge with pCMV-S (initial challenge). The remainder of spleen cells (containing 7.6 × 10^4 Env.28-Dimer+ CD8+ T cells) were transferred into naive non-irradiated Ly5.2+ mice. Recipient mice were subsequently challenged with 1 × 10^7 pfu vHBs4 (re-challenge) and sacrificed on day 8 after infection. Ly5.1+ or Ly5.1+ Env.28-Dimer+ CD8+ T cells were sorted separately from spleen cells. The same number (at least 3 × 10^4) of naive CD8+ T cells was also analyzed to verify the Gaussian distribution of the TCR repertoire. Purity of all isolated samples was at least 97% (data not shown). All procedures were carried out on individual mice. (B) TCR Vβ gene usage of different memory cell populations. The values plotted are averaged from four to six mice (mean ± SEM). (C) Representative TCR Vβ sub-repertoire profiles in the populations described above. Length in amino acids of the CDR3 regions, x-axis; normalized fluorescence intensity, y-axis, reflecting the number of clones using each Vβ/CDR3 length combination. The 15 panel sets within Vβ sub-repertoires are from an individual mouse. Results are representative of three independent experiments (n = 3–4 per experiment).
results, obtained under non-competitive conditions and using normalized cell numbers, support the finding that more recently generated memory cell populations have increased proliferative capacity.

Discussion

We have monitored the dynamics of an antigen-specific memory T cell population in vivo in response to repeated antigen challenge. By using serial adoptive transfer of isolated spleen cells, we were able to investigate endogenous polyclonal responses that approximate the natural host response to repeated infection. Consistent with previous results, we find that a recall response can include a population of newly primed memory T cells despite the presence of pre-existing memory T cells (10–12). Furthermore, this newly generated population contributes to the overall diversity of the memory T cell population (Fig. 3) and, importantly, expands more vigorously than an older population in a subsequent challenge (Figs. 4 and 5). A primary response during re-challenge therefore

Fig. 5. Functional capacities of primary and secondary memory. (A) Experimental design. Equivalent numbers of Ly5.1+ primary memory or secondary memory (1.4 × 10⁵) were adoptively transferred into Ly5.2+ mice (Group 1, primary memory; Group 2, secondary memory). Secondary memory cells were mixed with 1 × 10⁶ Ly5.1+ naive spleen cells to cell numbers constant. Mice that received only naive spleen cells were also analyzed (Group 3). Recipient mice were challenged with 5 × 10⁶ pfu vHBs.4 intra-peritoneally, and respective proliferative capacities (as measured by the number of splenic Env.28-Dimer+ CD8+ T cells) and anti-viral activities (as measured by virus titer in the ovaries) were determined on day 5. (B) Flow cytometry profiles of adjusted donor spleen cells before transfer (left) and 5 days after vHBs.4 challenge (right, gated on Ly5.1+ donor-derived cells). Numbers indicated in right upper quadrants are the percentage of Env.28-Dimer+ CD8+ T cell among all donor-derived CD8+ T cells. (C) Number of Ly5.1+ Env.28-Dimer+ CD8+ T cells in the spleen on day 5 after vHBs.4 challenge (n = 3–6, mean ± SEM). Statistical evaluations were carried out by the two-sample t-test (*P < 0.0001). (D) Control of vHBs.4 infection by primary or secondary memory following intra-peritoneal injection. Titers of vHBs.4 in the ovaries were determined on day 5 after challenge (n = 3–6, mean ± SEM, *P < 0.0001).
potentially makes a significant contribution in future antigen challenges, both qualitatively and quantitatively. This suggests that replenishing the supply of memory T cells may be important for the optimal response and maintenance of an antigen-specific memory T cell population. In addition, during the preparation of the manuscript, other groups reported a delayed conversion of secondary memory CD8+ T cells to CD62L+ phenotype (28, 29). This was proposed to be associated with reduced or delayed homing to lymph nodes, thus resulting in less interference with priming of naive T cells to generate new primary memory cells (28).

Previous studies have suggested that T cells compete for limited resources [such as access to APCs (30, 31) or cytokines such as IL-7 or 15 (32, 33)]. Indeed, we find that a primary CD8+ T cell response occurred only following quantitative expansion of pre-existing CD8+ T cells (Fig. 2). A likely interpretation is that pre-existing antigen-specific CD8+ T cells, which have better proliferative capacity than naive T cells, expand more vigorously upon antigen re-exposure and comprise the initial response. Only when antigen load overwhelms memory CD8+ T cells are functionally competent APCs available to naïve CD8+ T cells. Although generated under competition with older memory populations, primary CD8+ T cells were able to differentiate into fully functional effector, then memory, CD8+ T cells with the capacity for vigorous proliferation in a subsequent challenge (Figs 4 and 5 and Supplementary Figure 3, available at International Immunology Online).

Memory CD8+ T cells have been proposed to resemble stem cells in that both are long lived and have self-renewal capacity, allowing for the continual generation of descendant effector cells (2, 9, 34). In the memory maintenance phase, memory T cells self-renew through homeostatic proliferation in the presence of cytokines such as IL-15, IL-2 and IL-7, but independent of antigen and MHC (2, 33). A recent study also identified CD44loCD62LhiCD8+ T cells as candidate memory stem cells with the capacity to generate TCM, TEM and effector subsets while self-renewing in a graft-versus-host disease model (35). Given that we observe turnover within an antigen-specific T cell population, however, our results may indicate that memory T cells are not immortal. Rather, the memory T cell response to repetitive infection appears to be biased against older memory T cells, resulting in a population that is dynamically turned over in favor of new conscripts (Figs 4 and 5 and Supplementary Figure 3, available at International Immunology Online). This is consistent with other results that question the permanent sustainability of established memory T cells (29, 36). Collectively, these studies indicate a qualitative difference in memory T cells with repeated antigen challenge.

To our knowledge, ours is the first to indicate that memory T cell populations turn over in favor of memory T cells generated during a more recent challenge (Fig. 6).

Together with a quantitative contribution, we show that primary CD8+ T cells make a qualitative contribution to the memory T cell population by adding to its overall diversity. Previous studies of TCR repertoires within single antigen-specific populations that compared primary and secondary CD8+ T cells have had conflicting results. While some indicate a dramatic focusing of the secondary repertoire due to expansion of only a portion of the memory pool (37–39), others have shown little or no difference between primary and secondary populations (40–43). We propose that these two results are not mutually exclusive. Our experiments indicate that re-exposure to the same antigenic determinant causes the TCR repertoire of pre-existing memory T cell to skew significantly, yet overall TCR diversity is maintained, to some extent, through the addition of newly primed T cells. As a result, when thymic output continuously reconstitutes peripheral T cells, overall TCR diversity is maintained through the inclusion of these primary T cells in the memory T cell population (Fig. 3). Given that even a small gain in TCR repertoire diversity to a single viral epitope can result in higher resistance to pathogen (27, 44), this may afford the host greater versatility in controlling future infections.

The mechanism underlying this turnover remains to be determined. Recently, it was shown that the initial frequency of memory T cells can influence memory development (14).

![Fig. 6. Dynamic turnover model for a polyclonal memory T cell population with repeated antigen challenge. 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, 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.](https://academic.oup.com/intimm/article-abstract/19/1/105/712774/113)
This may be an important consideration in endogenous polyclonal responses, especially when compared with TCR Tg systems in which all monoclonal T cells with the same TCR affinity are expected to proliferate equally. Indeed, our VB usage and TCR repertoire analysis showed that memory T cell populations that consisted of several CDR3 length clones from an initial challenge yielded a predominant single CDR3 length profile in most VB genes after re-challenge. Deletion of some clones may add to the replicative burden of those that are selected. In addition, the procedure of two-step serial adoptive transfer might further force each memory T cell to divide more vigorously than in an undisturbed setting. These factors may cause memory T cell over-division, with consequent cell exhaustion and replicative senescence (29, 36). Yet another consideration is that we exclude memory T cells in compartments other than spleen (i.e. bone marrow, lymph nodes and/or peripheral non-lymphoid tissues), thereby neglecting memory T cell sub-populations that could also be sources of descendent effector T cells (45). However, this is unlikely to explain the turnover we observe in these populations. We used at least day 75 post-infection spleen cells (day 75 and day 260), long after the initial several weeks in which most phenotypical changes occur (46). Furthermore, it has been shown that memory T cells rapidly equilibrate into lymphoid and non-lymphoid organs following parabiosis (47). Although further experimentation is required to clarify the underlying mechanism for turnover, we believe that our results faithfully reproduce polyclonal responses to repeated antigen challenge in vivo.

A major goal of vaccination is to generate long-lived protective memory T cells. To produce large numbers of memory CD8+ T cells with propagation of high-affinity clones, current vaccination is widely based on prime-boost immunization. The precise mechanisms that control T cell expansion and lifespan during repeated exposure to complex pathogens is not well understood. Nonetheless, our data suggest that the ideal prime-boost strategy in vaccination 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.

In conclusion, we have demonstrated that a primary CD8+ T cell response during repeated antigen challenge contributes to the TCR diversity and proliferative potential of an antigen-specific memory T cell population. These results provide new insights that primary response rejuvenates memory T cell pool after multiple antigen challenges. Our results introduce another perspective on the heterogeneity of memory T cells: the number of antigen encounters.

Supplementary data

Supplementary Figures 1–3 are available at International Immunology Online.

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Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>CDR3</td>
<td>complementarity determining region</td>
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<td>HBV</td>
<td>hepatitis B virus</td>
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<td>ILH</td>
<td>intrahepatic lymphocyte</td>
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<tr>
<td>pfu</td>
<td>plaque-forming unit</td>
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<tr>
<td>TEM</td>
<td>central memory T cell</td>
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<tr>
<td>TEM</td>
<td>effector memory T cell</td>
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<td>Tg</td>
<td>transgenic</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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