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Heterogeneity of the Memory CD4 T Cell Response: Persisting Effectors and Resting Memory T Cells

Mojgan Ahmadzadeh, S. Farzana Hussain, and Donna L. Farber

Defining the cellular composition of the memory T cell pool has been complicated by an inability to distinguish effector and memory T cells. We present here an activation profile assay, using anti-CD3 and antigenic stimuli, that clearly distinguishes effector and memory CD4 T cells and defines subsets of long-lived memory CD4 T cells based on CD62 ligand (CD62L) expression. The CD62Llow memory subset functionally resembles effector cells, exhibiting hyper-responsiveness to antigenic and anti-CD3 mediated stimuli, high proliferative capacity, and rapid activation kinetics. The CD62Lhigh memory subset functionally resembles resting memory cells, exhibiting hyporesponsiveness to anti-CD3 stimuli, lower proliferative capacity, and slower activation kinetics. Our results indicate that the memory CD4 T cell pool is heterogeneous, consisting of persisting effectors and resting memory T cells. The Journal of Immunology, 2001, 166: 926–935.

The memory immune response, characterized by increased kinetics, higher levels of immune reactants, and efficacious clearance of Ag, is generally ascribed to the reactivation of Ag-specific memory T lymphocytes. Although it is unknown how memory T cells are generated, it has been shown that the majority of acutely activated effector T cells die in vivo (1), while a smaller proportion of previously activated T cells persist as memory T cells. Previous assumptions that these memory T cells represent a long-lived, resting subset have been clouded by recent findings of functional and/or phenotypic heterogeneity within the memory population. Functional heterogeneity based on differences in activation kinetics has been identified among virus-specific, memory CD8 T cells in humans and mice (2, 3) and among subsets of human memory CD4 and CD8 T cells that differ in expression of the chemokine receptor CCR7 (4). These kinetic variations in both the CD4 and CD8 memory T cell pool result in similar functional outcomes, and subsets of memory T cells exhibiting defined differences in activation or functional outcome have not been identified.

Long-lived memory CD4 and CD8 T cells also exhibit phenotypic heterogeneity based on surface expression of the lymph node-homing receptor, CD62 ligand (CD62L). Memory CD8 T cells that persist following viral or bacterial infection are heterogeneous for CD62L expression (5, 6). Similarly, CD62L heterogeneity has been observed in memory CD8 T cells generated from in vivo activation of TCR-transgenic T cells in adoptive hosts (6–9) and in persisting memory CD4 T cells generated by transfer of TCR-transgenic effector CD4 T cells into adoptive hosts (10, 11). Because CD62L expression (CD62Lhigh) is generally associated with a naive phenotype, and loss of CD62L expression (CD62Llow) is a hallmark of effector/memory subsets (12), CD62L heterogeneity among memory T cells remains unexplained at present. Whether CD62L expression delineates functional subsets within the memory T cell pool is also not known.

To characterize the cellular bases of heterogeneity within the memory T cell pool, it is essential to establish parameters that reliably distinguish effector from resting memory T cells, as it has been difficult to assess the life span and identity of these subsets in vivo due to similar functions and phenotypes. Functionally, effector and memory CD4 and CD8 T cells both mediate effector functions such as cytokine production and/or cytolyis, although effector T cells generally perform these functions with more rapid kinetics (13). Progress has recently been made in defining new cell surface phenotypes that distinguish effector and memory CD8 T cells (14); however, there is still no phenotypic marker that reliably distinguishes effector and memory CD4 T cells. We have been exploring alternate ways to distinguish effector and memory T cells and have recently shown that effector and memory CD4 T cells differ biochemically, as assessed by the tyrosine phosphorylation profile (10). This biochemical analysis requires a relatively large number of purified cells; therefore, a functional assay employing a small number of T cells to distinguish effector and memory CD4 T cell subsets would be highly advantageous.

In this study we demonstrate that effector and memory CD4 T cells can be clearly distinguished based on activation profile to different types of TCR-mediated stimuli, and we use these assays to analyze heterogeneity within the memory CD4 T cell pool. We previously found that effector CD4 T cells could maintain effector properties for several months in vivo based on biochemical analysis of the persisting population (10). However, we could not rule out that a resting memory population was generated and masked by a dominant effector profile. Here, we demonstrate that both conventional resting memory T cells and persisting effector CD4 T cells comprise the memory T cell pool and can be isolated based on CD62L expression. These two memory subsets mediate recall responses and produce effector cytokines, yet differ in overall proliferative capacity, kinetics of activation, activation profile, and spontaneous proliferation to MHC class II. These findings suggest that different subsets of memory CD4 T cells may play disparate roles in recall responses and may also have different requirements for maintenance in vivo.
Materials and Methods

Mice

BALB/c mice were obtained from National Cancer Institute Biological Testing Branch and were used between 8 and 12 wk of age. Hemaggutinin (HA)-TCR-transgenic mice (15) were bred as heterozygotes and maintained in the Microbiology Animal Facility at the University of Maryland (College Park, MD). MHC class II−/− mice (16) and recombinase-activating gene 2 (RAG2−/−) mice (17) on a BALB/c genetic background were purchased as breeding pairs from Taconic Farms (Germantown, NY) and bred and maintained in the animal facility under specific pathogen-free conditions.

Abs and reagents

The following Abs were purified from culture supernatants from hybridomas maintained in the laboratory: C363.29B (anti-CD3ε) (18), GK1.5 (anti-CD4) (19), anti-CD8 (Tib-105, American Type Culture Collection, Manassas, VA), 212.A1 (anti-CD4), anti-Fe-γR, anti-Thy1.2 (TIB 238), and anti-Mac-1 (TIB 128). The 6.5-anti-clonotype Ab (15) directed against the HA-TCR (rat IgG) hybridoma was purified and FITC conjugated as described previously (10). The following mAbs were purchased from PharMingen (San Diego, CA): PE-conjugated anti-CD45RB (clone 2.4G2), anti-Thy1.2 (clone 30-31H2), purified anti-mouse IgM and anti-mouse IgG, and anti-mouse IgM-coupled magnetic beads (Perceptive Biosystems, Cambridge, MA). CD4 T cells were fractionated into naive and memory subsets by positive and negative selection by MACS separation using anti-CD45RB conjugated anti-CD45RB (C363.16A, PharMingen) and streptavidin MACS MACS magnetic column. The resultant Thy1.2-compatible populations were purchased from Miltenyi Biotec (Auburn, CA). The HA peptide 110–119 of the sequence, SFERFEIPPK, was synthesized by Biopolymer Laboratory, University of Maryland School of Medicine.

Isolation of naive and memory HA-TCR subsets

The detailed procedure for isolation of mouse CD4 T cells and subsequent separation into CD45RBlow (memory) and CD45RBhigh (naive) subsets was detailed previously (21). Briefly, CD4 T cells (>90% pure) were isolated from HA-TCR splenocytes using immunomagnetic depletion with anti-CD8 and anti-CD4 magnetic beads followed by anti-rat IgG, anti-mouse IgG1, and antihuman IgM-coupled magnetic beads (Perceptive Biosystems, Cambridge, MA). CD4 T cells were then fractionated into naive and memory subsets by positive and negative selection by MACS separation using anti-CD45RB conjugated anti-CD45RB (C363.16A, PharMingen) and streptavidin MACS MACS magnetic columns (Miltenyi Biotec). The resultant CD45RBlow (memory) and CD45RBhigh (naive) populations were >95% CD4.

In vitro generation of effector cells

Ag-activated effector CD4 T cells were generated from HA-TCR CD4 T cells (1 × 10⁶ cells/ml) incubated with 5 μg/ml HA peptide and 3 × 10⁶ cells/ml T-depleted BALB/c splenocytes as APC in 24-well plates for 3–5 days at 37°C (10). For in vitro functional analyses, effector cells were centrifuged through Ficoll after 5 days to remove dead cells and contaminating accessory cells, washed in PBS, and resuspended in complete Clicks medium (10). Effector generated in this way were >95% pure and had no residual APC.

Proliferation and cytokine assays

CD45RBhigh, CD45RBlow, and in vitro generated effector subsets (50,000 cells/well) were incubated in flat-bottom 96-well plates with APC (150,000/well) in complete Clicks medium. Titrated amounts of either HA peptide or anti-CD3ε Ab were added. Cells were incubated at 37°C, proliferation was assessed after 24 and 48 h by the addition of 1 μCi [3H]thymidine (6.7 Ci/mmol)/well, and cells were harvested after 18 h using a Tomtec 96-well plate harvester (Wallac, Gaithersburg, MD). Radioactivity was quantitated using a MicroBeta TriLux plate scintillation counter (Wallac), and proliferation was expressed as the average of triplicates in which errors were consistently <10%. Supernatants from duplicate cultures set up for proliferation were collected after 24 and 48 h. IFN-γ in supernatants was measured by specific ELISA (Endogen, Cambridge, MA). Color reactions were read at 450 absorbance in an ELISA reader (Bio-Rad), and units per milliliter of IFN-γ were calculated by comparison to a known IFN-γ standard. For analysis of CD4 T cells and CD62L subsets from adoptive transfer recipient mice, assays were set up as described above, except that 37,500 T cells were added per well, and proliferation was assessed after 24–72 h in culture.

Adoptive transfers and cell purification

Effector CD4 T cells were purified through Ficoll (LSM, ICN, Costa Mesa, CA) after 3 days in culture, washed three or four times in PBS, and resuspended in 10⁶ cells/0.5 ml of PBS. Effector cells or equal numbers of purified resting HA-TCR CD4 T cells from naive mice were injected in 0.5 ml into the tail vein of RAG2−/− mice. Adoptive transfer recipient mice were sacrificed 8–13 wk post-transfer, and splenic CD4 T cells were isolated using MACS magnetic sorting. Briefly, splenocytes were first incubated at 37°C for 1 h to remove adherent cells. Nonadherent cells were collected and incubated with FcR blocking reagent (Miltenyi) for 15 min following anti-Thy1.2 microbeads (Miltenyi) for 10 min on ice. Cells were washed, resuspended in PBS/1% FCS, and positively selected over a MACS magnetic column.

FACS sorting and analysis

CD4 T cells from adoptive transfer recipient mice or HA-TCR mice were enriched by immunomagnetic depletion. Briefly, splenocytes were incubated with an Ab cocktail containing anti-Mac-1a, anti-FcγR, anti-Ly6G, and anti-MHC II followed by anti-rat IgG1, anti-mouse IgG1, and anti-mouse IgM-coupled magnetic beads. The cells were subsequently stained with FITC-conjugated anti-Thy1.2 and PE-conjugated anti-CD62L before sorting on an EPICS Elite ESP flow cell sorter (Coulter, Miami, FL). The resultant Thy1.2 CD62Llow and Thy1.2 CD62Lhigh sorted populations were 90–97% pure. For staining, cells were washed and resuspended in stain buffer (PBS, 5% FCS, and 0.05% sodium azide). Stained cells were analyzed using a FACSCalibur (Becton Dickinson, San Jose, CA) with CellQuest software.

Results

Generation and functional analysis of HA-specific effector CD4 T cells

To characterize effector CD4 T cells relative to naive and memory T cell counterparts, we first generated Ag-specific effector CD4 T cells from HA-TCR transgenic mice in which a large proportion of peripheral CD4 T cells express the transgene-encoded TCR specific for peptide 110–119 of influenza HA and I-Eα (15). We previously showed that activation of naive HA-TCR CD4 T cells for 5 days with HA peptide in the presence of splenic MHC class II+ APC resulted in activated cells bearing the functional and phenotypic attributes of differentiated effector CD4 T cells (10). We compared activation parameters of this effector CD4 T cell population relative to ex vivo naive (CD45RBhigh) HA-TCR CD4 T cell precursors by stimulating with different doses of HA peptide in the presence of APC. Overall, we found that effector cells exhibit peak proliferative responses between 24–48 h, whereas naive CD4 T cells exhibit maximal proliferation at 72 h (data not shown). As shown in Fig. 1A, at 48 h effector CD4 T cells proliferate extensively at all Ag doses, whereas naive T cells proliferate at a lower level than effectors only at higher Ag doses (see inset), consistent with similar findings by Iezzi (22). Effector CD4 T cells did not proliferate in response to HA peptide alone, confirming the lack of contaminating APC (Fig. 1A). Reconstitution of HA-TCR effectors with increasing doses of Ag also resulted in increasing levels of IFN-γ production, whereas naive HA-TCR CD4 T cells did not produce significant levels of IFN-γ even at high peptide doses (Fig. 1B). These results demonstrate that the in vitro-generated HA-TCR effector population is functionally hyper-responsive to antigenic stimulation compared with naive CD4 T cells, and that effector CD4 T cell proliferation, when measured at early time points, correlates with cytokine secretion.

Activation profile of naive, memory, and effector CD4 T cells

Although naive and effector CD4 T cells can be clearly distinguished by their response to antigenic stimulation (Fig. 1), functional differences between memory and effector CD4 T cells are not clearly defined. With the goal of defining functional parameters that delineate effector and memory CD4 T cells, we considered the striking differences in activation requirements to anti-CD3-mediated
stimulation exhibited by ex vivo naive and memory CD4 T cells (see Table I). We and others had previously demonstrated that murine memory CD4 T cells are hyporesponsive to soluble anti-CD3 Ab in the presence of MHC class II<sup>+</sup> splenic APC (anti-CD3/II<sup>+</sup> APC), whereas naive CD4 T cells are fully activated by this stimulus (21, 23) (see Table I). This memory CD4 T cell hyporesponsiveness is dependent on the CD4-HMC class II interaction (21, 23), as memory CD4 T cells can be activated by anti-CD3 presented by MHC class II<sup>−</sup> APC (anti-CD3/II<sup>−</sup> APC) (21). Given this novel memory CD4 T cell-specific activation property, we asked whether effector CD4 T cells would likewise exhibit hyporesponsiveness to anti-CD3/II<sup>+</sup> APC.

For these experiments we isolated naturally occurring memory and naive CD4 T cells from HA-TCR mice as defined by CD45RB isoform expression (20). The majority (>90%) of splenic CD4 T cells in HA-TCR mice that express the transgene-encoded TCR (clonotype 6.5) exhibit a characteristic CD45RB<sup>bright</sup> naive phenotype (10). However, a small proportion (between 5–8%) of 6.5<sup>+</sup> CD4 T cells express the CD45RB<sup>low</sup> memory phenotype (data not shown), presumably by priming via a second, endogenously expressed TCR as found in another TCR-transgenic mouse strain (24). We isolated the naturally occurring CD45RB<sup>low</sup> (memory) and CD45RB<sup>bright</sup> (naive) subsets from HA-TCR mice and analyzed these subsets phenotypically and functionally. The CD45RB expression profile of these purified subsets is shown in Fig. 2A. To ensure that these subsets functionally represented naive and memory CD4 T cells, we tested for the absence and the presence, respectively, of effector cytokine production in response to HA peptide Ag. As shown in Fig. 2B, CD45RB<sup>low</sup> (memory) HA-TCR CD4 T cells produce high levels of IFN-γ in response to HA peptide, whereas equivalent numbers of 6.5<sup>+</sup> CD45RB<sup>bright</sup> (naive) HA-TCR CD4 T cells produce negligible levels of this effector cytokine. These results demonstrate that the CD45RB<sup>low</sup> subset of HA-TCR CD4 T cells functionally represents memory T cells, thus enabling a novel comparison of endogenous HA-specific memory to HA-specific effectors.

To establish whether effector CD4 T cells exhibit hyporesponsiveness to anti-CD3/II<sup>+</sup> APC similar to memory counterparts, we cultured naive, effector, and memory HA-TCR CD4 T cells with anti-CD3/II<sup>+</sup> APC and performed a dose and kinetic analysis of their responses. As shown in Fig. 2C, at all doses and time points effector CD4 T cells exhibit the highest level of proliferation to anti-CD3/II<sup>+</sup> APC, compared with ex vivo HA-TCR naive and memory subsets. Although effectors show maximal proliferation after 24 h of stimulation and hyper-responsiveness to anti-CD3/II<sup>+</sup> APC at the lowest doses of anti-CD3 stimulation (0.01–0.1 μg/ml; Fig. 2C), by contrast, at all time points and doses of anti-CD3 Ab, memory CD4 T cells fail to exhibit a significant proliferative response. These results demonstrate that when stimulated with anti-CD3/II<sup>+</sup> APC, effector CD4 T cells are hyper-responsive, and memory CD4 T cells are hyporesponsive.

Although effector and memory CD4 T cells can be clearly distinguished by their responses to anti-CD3/II<sup>+</sup> APC, we wished to examine multiple functional parameters to obtain an activation profile that clearly defines naive, effector, and memory CD4 T cell subsets. We thus analyzed proliferative responses of HA-TCR naive, effector, and memory CD4 T cells to Ag/APC, anti-CD3/
II\(^+\) APC and anti-CD3/II\(^-\) APC (memory CD4 T cell hyporesponsiveness to anti-CD3/II\(^+\) APC is overcome by anti-CD3/II\(^-\) APC (21); see Table I), to obtain an activation profile. As shown in Fig. 2, two themes emerge when examining individual activation profiles of naive, effector, and memory CD4 T cells. First, naive, effector, and memory CD4 T cells exhibit distinct activation profiles to the three types of TCR stimuli. (Each activation profile is depicted on a separate graph, so that relative responses to Ag-vs anti-CD3 can be compared.) Naive CD4 T cells proliferate well in response to Ag/APC and exhibit higher proliferative responses to anti-CD3-mediated stimuli in the presence of II\(^+\) or II\(^-\) APC (probably due to the activation of nonclonotype T cells). Effector CD4 T cells exhibit high proliferative responses to Ag/APC and equally high proliferative responses to anti-CD3/II\(^+\) APC and anti-CD3/II\(^-\) APC (Fig. 2D, middle graph). Although ex vivo-derived memory (CD45RB\(^{low}\)) HA-TCR CD4 T cells proliferate in response to Ag/APC and anti-CD3/II\(^+\) APC, they do not exhibit substantial proliferation in response to anti-CD3/II\(^-\) APC (Fig. 2D, right-most graph). Thus, while both naive and effector CD4 T cells exhibit antigen-specific responses lower or equivalent to anti-CD3/II\(^+\) APC responses, memory CD4 T cells exhibit greater responses to Ag compared with anti-CD3/II\(^+\) APC.

The second theme that emerges from the activation assays in Fig. 2D is that naive, effector, and memory CD4 T cells exhibit profound differences in proliferative capacity. Each subset responded maximally to stimulation with anti-CD3/II\(^+\) APC, and comparison of these responses reveals that effectors have the highest proliferative capacity overall, followed by naive and then memory CD4 T cells. The hyper-responsiveness of effectors is independent of stimulation by residual Ag and/or APC, because effectors stimulated with either APC alone or anti-CD3 or HA peptide alone exhibited negligible proliferation (Fig. 2 and data not shown). These results indicate that analysis of the activation profile to cognate and noncognate stimuli and overall proliferative capacities can be used to unambiguously distinguish effector and memory CD4 T cells (see Table I).

**Analysis of memory generation in vivo**

Given our ability to distinguish effector and memory CD4 T cells based on activation profile, we next applied this assay to analyze the generation of long-lived memory T cells from effector CD4 T cells in vivo. We had previously demonstrated that transfer of HA-TCR effector CD4 T cells into sublethally irradiated BALB/c adoptive hosts resulted in the persistence of Ag-specific T cells for many months that mediated recall responses to Ag (10), consistent with findings in other adoptive transfer systems (25, 26). Although these cells appeared smaller in size than effectors and were long-lived, this persisting population exhibited an effector-specific biochemical pattern of tyrosine phosphorylation. These results suggested that a proportion of transferred effectors were maintained in vivo as persisting effector CD4 T cells (10).

In this study, we asked whether these persisting cells likewise exhibited an effector-specific or memory cell-specific activation profile. For these analyses, we transferred HA-TCR effectors into syngeneic RAG2\(^{−/−}\) adoptive hosts to remove the complication of endogenous T cells. We purified the persisting splenic CD4 T cells...
8–13 wk post-transfer and designated these cells as CD4^{ET} because they derived from mice that received transferred effector cells (see Table II). For comparison, equal numbers of resting HA-TCR CD4 T cells were transferred in parallel, and the cells persisting 8–13 wk in vivo were designated CD4^{NT} (see Table II) because they derived from mice that received resting HA-TCR CD4 T cells consisting predominantly of naive CD4 T cells.

In agreement with our previous results (10) the CD4^{ET} cells persisting in RAG2^{−/−} hosts were phenotypically different from the transferred effector CD4 T cells. In particular, the expression of CD25, while up-regulated in effector T cells relative to naive HA-TCR precursors (Fig. 3A) was down-regulated in the CD4^{ET} population, consistent with analogous adoptive transfer systems (25, 26). In addition, while naive HA-TCR CD4 T cells were primarily CD62L^{hi}, both Ag-activated effectors and CD4^{ET} persisting 8–16 wk in adoptive hosts were heterogeneous for CD62L expression, with two distinct populations of CD62L^{lo} and CD62L^{hi}. To determine whether CD4^{ET} cells represented resting memory CD4 T cells in terms of activation parameters, we asked whether these persisting cells likewise exhibited hyperresponsiveness to anti-CD3/II^{+} APC. We thus assayed the ability of CD4^{ET} cells 10 wk post-transfer to respond to different doses of anti-CD3 in the presence of II^{−} APC compared with CD4^{NT} that persisted 10 wk post-transfer. As shown in Fig. 3B, CD4^{ET} cells exhibit a high proliferative response to low doses of anti-CD3 Ab (0.1 and 1 μg/ml) compared with CD4^{NT} cells, which respond substantially only at the highest dose tested (1 μg/ml). These results indicate that the CD4^{ET} cells that persist in vivo resemble effector CD4 T cells in their hyper-responsiveness to anti-CD3/II^{+} APC, similar to in vitro generated effectors (Fig. 2C).

To further analyze the activation properties of the CD4^{ET} persisting population, we activated CD4^{ET} with Ag/APC, anti-CD3/II^{+} APC, or anti-CD3/II^{−} APC to obtain an activation profile as in Fig. 2D. The activation profile of CD4^{ET} cells in Fig. 3C demonstrates that CD4^{ET} cells proliferate at high levels in response to Ag/APC, anti-CD3/II^{+} APC, and anti-CD3/II^{−} APC similar to in vitro generated effector cells (Fig. 2D). Controls with APC, Ag, or anti-CD3 Ab alone yielded negligible proliferative responses, confirming the lack of contaminating Ag and APC in the purified CD4^{ET} population (Fig. 3B). From these results we conclude that at least a fraction of the persisting CD4^{ET} cells exhibit effector-specific activation profiles. This maintenance of effector-specific properties over time is consistent with our previous findings that CD4^{ET} cells derived from BALB/c adoptive hosts exhibited effector-specific biochemical profiles (10).

**Isolation and functional analysis of heterogeneous CD4^{ET} subsets**

Although the persisting CD4^{ET} cells exhibited a high proliferative capacity and hyper-responsiveness to anti-CD3/II^{+} APC similar to effector T cells, the presence of conventional resting memory T

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**Table II. Nomenclature used to designate T cell subsets**

<table>
<thead>
<tr>
<th>Cell Subset Designation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>CD4^{ET}</td>
<td>CD4 T cells persisting following transfer of effecter T cells into RAG2^{−/−} hosts</td>
</tr>
<tr>
<td>CD4^{NT}</td>
<td>CD4 T cells persisting following transfer of naive CD4 T cells into RAG2^{−/−} hosts</td>
</tr>
<tr>
<td>CD62L^{lo}, CD62L^{hi}</td>
<td>Subsets of CD4 T cells from naive HA-TCR mice differing in CD62L expression</td>
</tr>
<tr>
<td>CD4^{ET}</td>
<td>CD4^{ET} cells fractionated into subsets differing in CD62L expression</td>
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**FIGURE 3.** Activation requirements of HA-TCR CD4 T cells persisting in adoptive hosts. In vitro-generated effector HA-TCR CD4 T cells or resting HA-TCR CD4 T cells were i.v. transferred into RAG2^{−/−} hosts. Ten weeks post-transfer, the persisting splenic CD4 T cells were purified by positive selection with anti-Thy1.2 MACS magnetic beads. A. Expression of CD62L and CD25 by naive, effector, and CD4^{ET}. Purified HA-TCR CD4 T cells, 5 day in vitro-generated HA-TCR effector CD4 T cells, and the persisting CD4^{ET} population were triple stained for 6.5, CD4, and CD25, and the results shown are gated on CD4^{+} 6.5^{−} T cells. The CD4^{ET} phenotype was similar as measured 8–16 wk post-transfer. B. Dose response to anti-CD3/MHC class II^{+} APC of the persisting CD4^{ET} cells from hosts that received effector T cells compared with the persisting CD4^{NT} cells from hosts that received predominantly naive HA-TCR CD4 T cells 10 wk previously. Proliferation was assessed after 3 days. C. Activation profile of the persisting HA-TCR CD4 T cells (CD4^{ET}). Purified CD4^{ET} cells (50,000/well) were cultured with anti-CD3 (1 μg/ml) in the presence of 150,000 II^{+} APC or II^{−} APC or HA peptide (1 μg/ml) in the presence of II^{+} APC. Proliferation was assessed after 3 days. These results are representative of five transfer experiments.
cells that exhibit lower proliferative capacities and hyporesponsiveness to anti-CD3/II-1APC would be masked by dominant effector functions. The phenotypic heterogeneity in CD62L expression found in the CD4ET-persisting population (see Fig. 3A) suggested that the long-lived memory T cell pool may consist of more than one subset of CD4 T cells. To determine whether differences in CD62L expression delineated subsets of long-lived memory T cells that differed in function and/or activation profile, we sorted the persisting CD4ET cells into CD62Llo ET and CD62Lhi ET populations (histogram plots in Fig. 4A) for subsequent functional analysis. For comparison, we also sorted CD62L T cell subsets directly from purified fresh HA-TCR CD4 T cells that were predominantly naive (CD62LloN and CD62LhiN, refer to Table II for nomenclature). Although the CD62LhiN subset predominated in CD4 T cells derived from naive HA-TCR mice as previously reported (10), the proportion of the CD62LloET subset in CD4ET cells ranged from equivalent (see Fig. 4A) to up to 3 times greater than the CD62LhiET subset, when individual mice were examined (data not shown).

To determine whether both CD62LET subsets mediated recall responses to specific Ag, we measured IFN-γ secretion by CD62L ET and CD62L N subsets in response to Ag. As demonstrated in Fig. 4B, both CD62LET subsets secrete IFN-γ in response to antigenic stimulation compared with the negligible level of IFN-γ secreted by CD62L N subsets in response to antigenic stimulation. Interestingly, the level of IFN-γ in culture supernatants from CD62LloET cells is 2- to 3-fold higher than that in supernatants from CD62LhiET cells, although both express comparable numbers (~85%) of 6.5 cells (data not shown). To further examine the cytokine properties of the CD62L ET subsets, we performed a dose and kinetic analysis of Ag-specific cytokine production (Fig. 4C). As illustrated in Fig. 4C, CD62LloET produces a higher level of IFN-γ with faster kinetics than the CD62LhiET subset. After 30 h in culture with Ag/APC, CD62LloET produces...
significantly different levels of IFN-γ, while the CD62Lh⁺ET subset does not produce appreciable IFN-γ levels. After 48 h in culture, however, the CD62Lh⁺ET subset produces substantial levels of IFN-γ, although this level is still one-third of that produced by CD62LloET. These results establish that both CD62L⁺ET subsets mediate recall responses, yet differ in the level and kinetics of effector cytokine production, with the CD62LloET subset exhibiting higher levels of IFN-γ production compared with the CD62Lh⁺ET subset.

Because the CD62L⁺ET subsets that persisted in vivo differ in kinetics of activation similar to previous distinctions between effector and resting memory T cells (27), we asked whether these CD62L⁺ET subsets could likewise be distinguished based on activation profile, demonstrated here to delineate effector and memory T cells (27). We thus measured the proliferative response of sorted CD62L⁺ET subsets to Ag or anti-CD3 Ab in the presence of MHC class II⁺ APC (II⁺ APC) and anti-CD3 in the presence of II⁺ APC or II⁻ APC was assessed after 3 days. A, Proliferation of CD62Llo ET and CD62Lh⁺ ET subsets isolated 11 wk post-transfer to HA peptide/MHC class II⁺ APC (II⁺ APC) and anti-CD3 in the presence of II⁺ APC or II⁻ APC was assessed after 3 days. B, Proliferation kinetics of CD62L⁺ET subsets in response to stimulation with 1 μg/ml anti-CD3 Ab in the presence of II⁺ APC or II⁻ APC. Proliferation was assessed after 48 and 72 h in culture. These results are representative of five sorting experiments.

During the course of functional analyses of the sorted CD62L⁺ET subsets, we also found that spontaneous proliferation in the presence of II⁺ APC differs between CD62L⁺ET subsets. As shown in Fig. 6, only the CD62LloET population exhibited significant background proliferation in response to II⁺ APC alone, whereas the CD62Lh⁺ET subset and both CD62L⁺N subsets sorted from fresh naive HA-TCR CD4 T cells exhibited negligible background proliferation to II⁺ APC. This CD62LloET-specific proliferation to II⁺ APC was consistently present in all the sorting experiments performed. Interestingly, neither the CD62L⁺ET nor the CD62L⁺N subset exhibited background proliferation to II⁺ APC, indicating that the proliferation in response to II⁺ APC most likely derives from interaction with MHC class II⁺ and endogenous peptide. We also found that in vitro-generated effectors, but not ex vivo-derived memory CD4 T cells defined by CD45RBlow expression, proliferate in response to MHC class II⁺ but not MHC class II⁻ APC (data not shown), suggesting that MHC class II-dependent turnover may be an effector-specific property.

**Discussion**

Recent findings of heterogeneity in the memory T cell pool in humans and mice suggest that more than one type of previously activated T cell may mediate recall responses. In this study, we analyzed the cellular composition of the memory CD4 T cell pool based on novel activation criteria shown here to distinguish effector and memory CD4 T cells. These activation criteria were found to delineate subpopulations of memory CD4 T cells that differed in CD62L expression, with the CD62Llo memory population resembling effector T cells and the
CD62Lhi memory population resembling resting memory T cells. Based on these observations, we conclude that the memory CD4 T cell pool consists of persisting effectors and resting memory T cells.

It has been difficult to follow the differentiative fate of effector cells in vivo and characterize memory T cell heterogeneity due to the lack of phenotypic markers and functional assays that reliably distinguish effector and memory T cells. Cell surface phenotypes have been shown to delineate effector and memory CD8 T cells in mice and humans (14, 28); however, clear-cut phenotypic or functional differences between effector and memory CD4 T cells have not yet been reported. To characterize the cells that comprise the memory CD4 T cell pool, we first established novel functional and activation differences between effector and memory CD4 T cells. As our standard for resting memory CD4 T cells generated in vivo, we used the CD45RBhi subset of splenic CD4 T cells, because this subset bears the phenotypic and functional properties of memory CD4 T cells (29). As presented here, ex vivo-derived memory CD4 T cells can be clearly distinguished from effector CD4 T cells based on activation profile to antigenic and anti-CD3-mediated stimuli. Specifically, effector CD4 T cells exhibit a greater proliferative capacity than memory CD4 T cells and are hyper-responsive to anti-CD3/II δ-APC and Ag, whereas memory CD4 T cells are hyporesponsive to anti-CD3/II δ-APC relative to Ag.

The differences in activation parameters identified here suggest that effector and memory CD4 T cells signal differently through the TCR/CD3 complex. We have previously demonstrated striking differences in the tyrosine phosphorylation profile of naive, effector, and memory CD4 T cells (10) and in proximal kinase phosphorylation in ex vivo derived naive and memory subsets (30). Differences in the expression of CD8-associated p56 \(^{\text{ck}}\) kinase have also been identified in naive vs effector/memory CD8 T cells (31). These functional and biochemical disparities suggest that alterations in the TCR-coupled signaling machinery control the distinct functional properties of naive, effector, and memory T cells. We are currently examining both proximal and distal TCR-mediated signal intermediates in these three subsets to address this hypothesis.

The identification of a functional assay to distinguish between effector and memory CD4 T cells enabled analysis of the differentiative fate of effector CD4 T cells in vivo and the cellular composition of the memory T cell pool. The memory T cell pool is defined here as the Ag-specific HA-TCR CD4 T cells that persist 8–13 wk following transfer of HA-TCR effectors into RAG2 \(^{-/-}\) adoptive hosts, analogous to other CD4 and CD8 adoptive transfer systems (25, 26, 32). By applying our activation profile assay, we found that the persisting memory population (designated CD4ET) exhibited an effector-specific activation profile in both its high proliferative capacity and its hyper-responsiveness to anti-CD3/II δ-APC. This finding was consistent with our previous results demonstrating that CD4ET exhibited an effector-specific biochemical profile (10). When taken together, our data suggest that effector CD4 T cells may persist for long periods of time in vivo, longer than previous estimates of effector T cell life span (33), yet consistent with recent findings that effector B cells or plasma cells are likewise long-lived in vivo (34).

We and others have found that the memory T cell population persisting after adoptive transfer of effectors was heterogeneous for the expression of CD62L (7, 10, 25). CD62L heterogeneity has likewise been observed in memory T cells that persist following viral (6, 35) or bacterial (5) infection. We found that the CD62Llo and CD62Lhi subsets differed strikingly in activation profile, proliferative capacity, and activation kinetics, similar to distinctions between effector and resting memory CD4 T cells, respectively. The association of effector-like functions with CD62Llo phenotype has been suggested in studies in which splenic LCMV-specific memory CD8 T cells heterogeneous for CD62L expression exhibited more rapid effector function than lymph node memory CD8 T cells that were primarily CD62Lhi (35). Similarly, the CD62Llo fraction of long-lived Sendai virus-specific memory CD8 T cells was found to exhibit a higher proliferative capacity in vitro than the CD62Lhi fraction, as assessed by CFSE staining (6). Although loss of CD62L expression is typically associated with effector/memory function, CD62Lhi memory CD4 T cells have been found in unmanipulated mice (36) and were found to predominate in aged mice (37). These results suggest that resting memory T cells may reacquire CD62L expression over time in vivo.

Subsets of human memory CD4 T cells have recently been isolated by Lanzavecchia and colleagues based on expression of the chemokine receptor CCR7 (4). The CCR7 \(^+\) memory subset was designated as effector-memory (TEm) due to rapid activation kinetics, whereas the CCR7 \(^+\) subset was designated central memory (Tcm) due to slower activation kinetics. Although the Tcm subset is predominantly CD62Lhi (4) and may correspond to the mouse CD62Lhi resting memory subset identified here, the TEm subset is heterogeneous for CD62L expression (4). Taken together, our results and those reported by Lanzavecchia suggest that both the CD62L homing receptor and the CCR7 chemokine receptor may be differentially expressed on memory T cell subsets. Once the murine CCR7-specific reagents are available, we can assess the
pattern of CCR7 expression in the mouse CD62L memory subsets and determine whether additional memory subsets can be defined by coordinate expression of these two markers.

Current models for the generation of memory T cells do not take into account heterogeneity of the memory T cell pool (38). The linear model predicts the generation of resting memory T cells directly from effectors that have reverted to the resting state. By contrast, a divergent model predicts distinct generation of effectors and memory T cells directly from a naive T cell precursor. We present in Fig. 7 a modified model for the generation of memory in which the two memory subsets diverge either from an effector intermediate (scheme 1) or from a pre-effector intermediate (scheme 2). Two other possibilities that cannot be ruled out are that resting memory T cells derive from persisting effectors (Fig. 7, scheme 3) and, conversely, persisting effectors may derive from a memory intermediate as has been suggested by Lanzavecchia (4). Following the development of each memory subset in vivo will enable identification of the cellular precursor for each memory T cell subset.

Generation of these two memory subsets may be differentially regulated. Zinkernagel and colleagues have shown that the same Ag delivered by a viral or bacterial pathogen elicited memory CD8 T cells differing in protective capacity, longevity, and activation kinetics (39), suggesting that the type and duration of the anamnestic response are affected by the activating stimulus. Experiments are currently underway to determine whether the initial activation condition affects the proportion of CD62Llo and CD62Lhi subsets in the memory CD4 T cell pool, an important consideration for vaccine design where a certain type of memory response may have protective advantage.

What is the purpose of these two subsets of memory T cells in anamnestic responses? It is feasible that each memory subset provides a unique role in memory responses in both the type of protection provided and the type of APC they encounter or activate. The persisting effector population with its rapid recall response and effector function may form the first line of protection in peripheral tissues. It has been shown that the CD62Llo effector subset rapidly migrates to inflammatory sites such as bronchial tissue to eradicate influenza infection (40) or to tumor sites to combat fibrosarcoma (41). In the tissues the CD62Llo subset may also recruit and prime tissue macrophages and immature dendritic cells. By contrast, the CD62Lhi resting memory subset migrates to secondary lymph nodes to be reprimed by resident APC (40), enabling them to provide help to B cells. We are currently examining the role of each CD62L memory subset in recall responses and their potential protective capacity in vivo.

In addition to playing distinct roles in recall responses, these two memory subsets may have different life spans and/or requirements for long-term maintenance in vivo. Although it has recently been demonstrated that CD4 T cell memory can persist in the absence of MHC class II (42), it is unclear whether this phenomenon applies to both subsets of memory T cells. Our results that only the CD62Llo memory subset exhibited spontaneous proliferation to MHC class II in the absence of cognate Ag suggests that subsets of memory CD4 T cells may be differentially maintained via MHC class II-dependent turnover. These subsets may also be differentially maintained in the presence of Ag. Although many studies have focussed on the question of memory persistence in the absence of Ag (43–45), in vivo Ag may indeed persist for an extended period of time depending on the nature of the pathogen or Ag (46). Whether the longevity of persisting effectors is dependent on the presence or the absence of Ag remains to be determined.

In conclusion, the results presented here demonstrate that the memory CD4 T cell pool can be subdivided into two subsets that differ in phenotype and multiple functional parameters. Elucidation of the generation and maintenance of these subsets in vivo and their roles in protective immunity can lead to improved vaccine strategies to generate the appropriate type of anamnestic response.

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