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

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

*J Immunol* 2001; 166:926-935; doi: 10.4049/jimmunol.166.2.926
http://www.jimmunol.org/content/166/2/926

References
This article cites 46 articles, 29 of which you can access for free at:
http://www.jimmunol.org/content/166/2/926.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/PUBLICATIONS/PI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
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 hypo-responsiveness 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 cytolysis, 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.

Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD 20742

Received for publication August 8, 2000. Accepted for publication October 24, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by National Institutes of Health Grant R29AI42092 (to D.L.F.).

2 Address correspondence and reprint requests to Dr. Donna L. Farber, Department of Surgery, University of Maryland, MSTF Building, Room 400, 685 West Baltimore Street, Baltimore, MD 21201. E-mail address: dfarber@smail.umaryland.edu

3 Abbreviations used in this paper: CD62L, CD62 ligand; HA, hemagglutinin; RAG2, recombinase-activating gene 2.
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. Hemagglutinin (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-CD3e) (18), GK1.5 (anti-CD4) (19), anti-CD8 (Tib-105, American Type Culture Collection, Manassas, VA), 212.A1 (anti-I-A$^b$), anti-Fc$\gamma$R (clone 2.4G2), anti-Thy1.2 (TIB 238), and anti-Mac-1$\alpha$ (TIB 128). The 6.5 anti-clonotypic 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- and biotin-conjugated anti-CD45RB (clone C363.16A) (20), PE-conjugated anti-CD62L (clone MEL-14), FITC-conjugated anti-CD25 (clone 7D4), FITC-conjugated Thy-1.2 (clone 30-H12), purified anti-mouse Ly-6G (clone RB6-8C5, and PE-conjugated anti-CD4. Quantum Red-conjugated anti-CD4 (clone H129.19) was purchased from Sigma (St. Louis, MO). The MACS anti-CD90 (Thy1.2) microbeads were purchased from Miltenyi Biotec (Auburn, CA). The HA peptide 110–119 of the sequence, SFERFEIFPK, 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) populations was detailed previously (21). Briefly, CD4 cells (>90% pure) were isolated from HA-TCR splenocytes using immunomagnetic depletion with anti-CD8 and anti-CD4 Abs followed by anti-rat IgG-, 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 biotin-conjugated anti-CD45RB (C363.16A, Pharmingen) and streptavidin MACS magnetic beads (Miltenyi Biotec). The resultant CD45RBlow (memory) and CD45RBhigh (naive) populations were >95% pure.

In vitro generation of effector cells

Ag-activated effector CD4 T cells were generated from HA-TCR CD4 T cells (1 × 10$^6$ cells/ml) incubated with 5 μg/ml HA peptide and 3 × 10$^6$ 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 cells 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-CD3e Ab were added. Cells were incubated at 37°C, proliferation was assessed after 24 and 48 h by the addition of 1 μCl [H]thymidine (6.7 Ci/mmol/well), and cells were harvested after 18 h using a Tomtec 96-plate well harvester (Wallac, Gaithersburg, MD). Radioactivity was quantitated using a Microbeta Tri-luxe plate scintillation counter (Wallac). 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 10$^7$ 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 followed by anti-Thy 1.2 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-1+, anti-FcγR, anti-Ly6G, and anti-MHC II followed by anti-rat IgG-, anti-mouse IgG-, and anti-mouse IgM-coupled magnetic beads. The cells were subsequently stained with FITC-conjugated anti-Thy 1.2 and PE-conjugated anti-CD62L before sorting on an EPICS Elite ESP flow cell sorter (Coulter, Miami, FL). The resultant Thy 1.2$^{CD62L^{−/−}}$ and Thy 1.2$^{CD62L^{high}}$ 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-E$^d$ (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). Restimulation 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+ splenic APC (anti-CD3/II+ 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/MHC class II interaction (21, 23), as memory CD4 T cells can be activated by anti-CD3 presented by MHC class II+ APC (anti-CD3/II+ 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+ 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 CD45RBhigh naive phenotype (10). However, a small proportion (between 5–8%) of 6.5+ CD4 T cells express the CD45RBlow 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 CD45RBlow (memory) and CD45RBhigh (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, CD45RBlow (memory) HA-TCR CD4 T cells produce high levels of IFN-γ in response to HA peptide, whereas equivalent numbers of 6.5+ CD45RBhigh (naive) HA-TCR CD4 T cells produce negligible levels of this effector cytokine. These results demonstrate that the CD45RBlow 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+ APC similar to memory counterparts, we cultured naive, effector, and memory HA-TCR CD4 T cells with anti-CD3/II+ 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+ 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+ APC at the lowest doses of anti-CD3 stimulation (0.01–0.1 μg/ml), naive CD4 T cells show increased proliferation at 48 h only at higher doses of anti-CD3 (1–5 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+ 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+ 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+

---

Table I. Activation requirements of naive, effector, and memory CD4 T cells

<table>
<thead>
<tr>
<th>Activation Stimulus</th>
<th>Naive&lt;sup&gt;a&lt;/sup&gt; (CD45RB&lt;sup&gt;bhi&lt;/sup&gt;)</th>
<th>Memory&lt;sup&gt;b&lt;/sup&gt; (CD45RB&lt;sup&gt;blo&lt;/sup&gt;)</th>
<th>Effector&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag/MHC class II+ APC</td>
<td>++</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>Anti-CD3 Ab/MHC class II+ APC</td>
<td>+++</td>
<td>–</td>
<td>++++</td>
</tr>
<tr>
<td>Anti-CD3 Ab/MHC class II+ APC</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

<sup>a</sup>Refs. 21 and 22.  
<sup>b</sup>Refs. 21 and 29.  
<sup>c</sup>Results shown in this study.  
<sup>d</sup>Ref. 22 and this study.

---

FIGURE 1. Functional analysis of in vitro generated effector HA-TCR CD4 T cells. HA-TCR effector CD4 T cells generated from HA-TCR CD4 T cells cultured with 5 μg/ml HA peptide and mitomycin C-treated BALB/c APC for 5 days were washed and recultured with different doses of HA peptide with or without splenic APC. Parallel cultures were set up with naive (CD45RB<sup>bhi</sup>) CD4 T cells freshly isolated from HA-TCR mice. A, Proliferation of naive (open symbols) and effector (closed symbols) HA-TCR CD4 T cells assessed after 48 h in culture, normalized for equivalent numbers of clonotype (6.5<sup>+</sup>) CD4 T cells. The inset gives an enlarged view of naive CD4 T cell proliferation to different peptide Ag doses. Results are averages of triplicates with variances <10%. B, Production of IFN-γ from equivalent numbers of 6.5<sup>+</sup> naive and effector HA-TCR CD4 T cells stimulated with different doses of HA peptide. IFN-γ was quantified by specific ELISA from 24-h culture supernatants. Results are representative of five different experiments.
IIλ' APC and anti-CD3/IIλ' APC (memory CD4 T cell hyperresponsiveness to anti-CD3/IIλ' APC is overcome by anti-CD3/IIλ' APC (21); see Table I), to obtain an activation profile. As shown in Fig. 2D, 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 IIλ' or IIλ' 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/IIλ' APC and anti-CD3/IIλ' APC (Fig. 2D, middle graph). Although ex vivo-derived memory (CD45RBlo) HA-TCR CD4 T cells proliferate in response to Ag/APC and anti-CD3/IIλ' APC, they do not exhibit substantial proliferation in response to anti-CD3/IIλ' APC (Fig. 2D, right-most graph). Thus, while both naive and effector CD4 T cells exhibit antigenic responses lower or equivalent to anti-CD3/IIλ' APC responses, memory CD4 T cells exhibit greater responses to Ag compared with anti-CD3/IIλ' 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/IIλ' 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 CD4ET 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 CD4NT (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 CD4ET cells persisting in RAG2<sup>−/−</sup> 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 CD4ET population, consistent with analogous adoptive transfer systems (25, 26). In addition, while naive HA-TCR CD4 T cells were primarily CD62L<sup>lo</sup> (50,000/well) were cultured with anti-CD3 (1 μg/ml) in the presence of MHC class II<sup>−/−</sup> hosts. The activation profile of CD4ET cells in Fig. 3D demonstrates that CD4ET cells proliferate at high levels in response to Ag/APC, anti-CD3/II<sup>+</sup> APC, and anti-CD3/II<sup>+</sup> APC 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 CD4ET population (Fig. 3B). From these results we conclude that at least a fraction of the persisting CD4ET cells exhibit effector-specific activation profiles. This maintenance of effector-specific properties over time is consistent with our previous findings that CD4ET cells derived from BALB/c adoptive hosts exhibited effector-specific biochemical profiles (10).

**Isolation and functional analysis of heterogeneous CD4ET subsets**

Although the persisting CD4ET cells exhibited a high proliferative capacity and hyper-responsiveness to anti-CD3/II<sup>+</sup> APC similar to effector T cells, the presence of conventional resting memory T cells in terms of activation parameters, we asked whether these persisting cells likewise exhibited hyperresponsiveness to anti-CD3/II<sup>+</sup> APC. We thus assayed the ability of CD4ET cells 10 wk post-transfer to respond to different doses of anti-CD3 in the presence of II<sup>+</sup> APC compared with CD4NT that persisted 10 wk post-transfer. As shown in Fig. 3B, CD4ET cells exhibit a high proliferative response to low doses of anti-CD3 Ab (0.1 and 1 μg/ml) compared with CD4NT cells, which respond substantially only at the highest dose tested (1 μg/ml). These results indicate that the CD4ET cells that persist in vivo resemble effector CD4 T cells in their hyper-responsiveness to anti-CD3/II<sup>+</sup> APC, similar to in vitro generated effectors (Fig. 2C).

To further analyze the activation properties of the CD4ET persisting population, we activated CD4ET with Ag/APC, anti-CD3/II<sup>+</sup> APC, or anti-CD3/II<sup>+</sup> APC to obtain an activation profile as in Fig. 2D. The activation profile of CD4ET cells in Fig. 3C demonstrates that CD4ET cells proliferate at high levels in response to Ag/APC, anti-CD3/II<sup>+</sup> APC, and anti-CD3/II<sup>+</sup> APC 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 CD4ET population (Fig. 3B). From these results we conclude that at least a fraction of the persisting CD4ET cells exhibit effector-specific activation profiles. This maintenance of effector-specific properties over time is consistent with our previous findings that CD4ET cells derived from BALB/c adoptive hosts exhibited effector-specific biochemical profiles (10).
cells that exhibit lower proliferative capacities and hyporesponsiveness to anti-CD3/II APC 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 CD62Llo ET and CD62Lhi ET subsets in response to antigenic stimulation compared with the negligible level of IFN-γ secreted by CD62LloN subsets in response to antigenic stimulation. Interestingly, the level of IFN-γ in culture supernatants from CD62Llo ET cells is 2- to 3-fold higher than that in supernatants from CD62Lhi ET cells, although both express comparable numbers (~85%) of 6.5 cells (data not shown). To further examine the cytokine properties of the CD62LET 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...
genic and anti-CD3-mediated stimuli. The CD62Llo ET subset pro-

eresponsive to anti-CD3/II^+ APC or II^- APC was assessed after 3 days. B, Proliferation kinetics of CD62L^hi 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.

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 (see Fig. 2). We thus measured the proliferative response of sorted CD62L^ET subsets to Ag or anti-CD3 Ab in the presence of II^+ APC or II^- APC. As shown in Fig. 5A, the CD62Llo ET and CD62L^hi ET subsets exhibit disparate activation profiles to antigenic and anti-CD3-mediated stimuli. The CD62Llo ET subset proliferates vigorously in response to Ag/ APC or anti-CD3 in the presence of II^+ or II^- APC. By contrast, the CD62L^hi ET subset is hyporesponsive to anti-CD3/II^- APC, while it proliferates well in response to Ag/ APC and anti-CD3/II^+ APC, similar to the activation profile observed with ex vivo isolated resting memory CD4 T cells (see Fig. 2C).

To further analyze responses of the CD62L^ET subsets to non-
cognate stimuli, we performed a kinetic analysis of proliferation in response to anti-CD3/II^+ APC and anti-CD3/II^- APC after 48 and 72 h in culture. As shown in Fig. 5B, CD62Llo ET proliferation in response to either anti-CD3/II^+ APC or anti-CD3/II^- APC increases ~2-fold between 48 and 72 h. By contrast, CD62L^hi ET proliferation in response to anti-CD3/II^- APC is negligible at 48 h and remains low after 72 h in culture, while proliferation in response to anti-CD3/II^+ APC at 48 h is comparable to that in the CD62Llo ET subset and doubles at 72 h (Fig. 5B, right). Taken together, the results in Fig. 5 demonstrate that the sorted CD62Llo ET and CD62L^hi ET subsets exhibit distinct activation profiles. The CD62Llo ET subset has a higher proliferative capacity and is hyper-responsive to stimulation by Ag/ APC, anti-CD3/II^+ APC, and anti-CD3/II^- APC similar to effector CD4 T cells, whereas the CD62L^hi ET subset has a lower proliferative capacity overall and is hyporesponsive to stimulation by anti-CD3/II^- APC, similar to resting memory CD4 T cells.

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 CD62Llo ET population exhibited significant background proliferation in response to II^- APC alone, whereas the CD62L^hi ET subset, and both CD62LN subsets sorted from fresh naive HA-TCR CD4 T cells exhibited negligible background proliferation to II^- APC. This CD62Llo ET-specific proliferation to II^- APC was consistently present in all the sorting experiments performed. Interestingly, neither the CD62L^ET nor the CD62LN 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 CD45RB^low 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
Differences in the expression of CD8-associated p56 phosphorylation in ex vivo derived naive and memory subsets (30) and in proximal kinase phosphorylations (10) and in proximal kinase phosphorylations of naive, effector to anti-CD3/II 1 stimuli. Specifically, effector CD4 T cells exhibit a greater proliferative capacity, and its hyper-responsiveness to anti-CD3/II1 APC.

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 TCR-coupled signaling machinery control the distinct functional properties 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 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/II1 APC.

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 TCR-coupled signaling machinery control the distinct functional properties of naive, effector, and 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/II1 APC and Ag, whereas memory CD4 T cells are hyporesponsive to anti-CD3/II1 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 TCR-coupled signaling machinery control the distinct functional properties of naive, effector, and 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/II1 APC and Ag, whereas memory CD4 T cells are hyporesponsive to anti-CD3/II1 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 TCR-coupled signaling machinery control the distinct functional properties of naive, effector, and 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/II1 APC and Ag, whereas memory CD4 T cells are hyporesponsive to anti-CD3/II1 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 TCR-coupled signaling machinery control the distinct functional properties of naive, effector, and 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/II1 APC and Ag, whereas memory CD4 T cells are hyporesponsive to anti-CD3/II1 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 TCR-coupled signaling machinery control the distinct functional properties of naive, effector, and 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/II1 APC and Ag, whereas memory CD4 T cells are hyporesponsive to anti-CD3/II1 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 TCR-coupled signaling machinery control the distinct functional properties of naive, effector, and 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/II1 APC and Ag, whereas memory CD4 T cells are hyporesponsive to anti-CD3/II1 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 TCR-coupled signaling machinery control the distinct functional properties of naive, effector, and 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/II1 APC and Ag, whereas memory CD4 T cells are hyporesponsive to anti-CD3/II1 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 TCR-coupled signaling machinery control the distinct functional properties of naive, effector, and 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/II1 APC and Ag, whereas memory CD4 T cells are hyporesponsive to anti-CD3/II1 APC relative to Ag.
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 naïve 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 (44). 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.

Acknowledgments

We thank Karen M. Wolcott (Biomedical Instrumentation Center, Uniformed Services University of the Health Sciences, Bethesda, MD) for excellent assistance with cell sorting, and Dr. Gregg Hadley (Department of Surgery, University of Maryland Medical School, Baltimore, MD) and Dr. Sandeep Krishnan (University of Maryland, College Park, MD) for critical reading of this manuscript.

References


