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Effector CD4 T Cells Are Biochemically Distinct from the Memory Subset: Evidence for Long-Term Persistence of Effectors In Vivo

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Memory T cell responses are believed to be mediated by long-lived memory T cells that arise directly from a subset of short-lived, activated effector T cells that have reverted to the resting state. Although widely accepted, definitive proof that memory T cells arise from effectors is lacking because of the inability to reliably distinguish these subsets based on known phenotypic or functional parameters. We have used a biochemical approach to distinguish effector and memory CD4 T cell subsets and follow the differentiative fate of effector cells in vivo. When examined biochemically, effector and memory CD4 T cells are strikingly distinct and exhibit qualitative and quantitative differences in tyrosine phosphorylation. These effector-specific patterns were identical in effectors derived either from naive CD4 T cells (primary effectors) or memory CD4 T cells (memory effectors). To monitor the fate of effector cells in vivo, Ag-activated CD4+ TCR-transgenic T cells were transferred into irradiated BALB/c mice. These TCR-transgenic CD4 T cells persisted in adoptive hosts for several months, gave a recall response to Ag, yet exhibited effector-specific biochemical profiles. These results suggest that a subset of effector CD4 T cells can persist in vivo and contribute to long-term immunity by mediating secondary immune responses. The Journal of Immunology, 1999, 163: 3053–3063.

Long-term immunity to pathogens previously introduced through infection or vaccination is believed to reside in memory T and B lymphocytes that persist in a resting state over the lifetime of an individual. When reactivated, memory lymphocytes mediate the anamnestic immune response that qualitatively and quantitatively-surpasses a primary immune response to Ags not previously seen. While the greater effectiveness of this memory immune response is the basis for the success of many vaccines, mechanisms governing the generation of immunological memory have not yet been elucidated.

During an initial encounter with Ag, naive Ag-specific lymphocytes proliferate and differentiate to become activated effector cells. B lymphocyte effectors are terminally differentiated Ab-secreting plasma cells, and T lymphocyte effectors, specifically effector CD4 T cells, produce multiple cytokines to coordinate the adaptive immune response. The previously held dogma states that most of these activated effector T and B cells die after a brief life span (1–3); however, recent compelling data have demonstrated that for B cells, Ag-specific plasma effector cells can persist and continue to secrete Abs over the lifetime of a mouse (4). This finding suggests that B cell-mediated memory immune responses characterized by high Ab titers, result from both persisting effector B cells and reactivated memory B cells.

Mechanisms underlying the generation and perpetuation of T cell-mediated memory immune responses are not known. A major impediment in studying T cell memory is that effector and memory T lymphocytes cannot reliably be distinguished based on known phenotypic and functional parameters (5) (see below). Because memory T cells resemble effectors, the favored model states that memory T cells arise directly from activated effectors that have reverted (via an unknown mechanism) to the resting state. An alternate model, however, states that memory T cells arise directly from activation of naive T cells independent of effector T cells. For B cells, the memory subset can be distinguished from effector cells based on surface expression of class-switched Ig isotypes and MHC class II (6). Based on these distinguishing features, it was found that generation of memory B cells occurs distinct from plasma cell generation (50). Thus, assessing whether T cells follow linear or divergent pathways for generation of memory and whether T cell-mediated memory can likewise result from long-lived effector cells critically depends on our ability to distinguish effector and memory T cells.

The phenotypic markers used to distinguish resting naive and memory CD4 T cells do not reliably differentiate between effector and memory subsets. These markers include the adhesion molecules CD44 (7, 8) and LFA-1 (8, 9), the homing receptor CD62L, and isoforms of the CD45 glycoprotein (10–12). In general, mouse effector and memory CD4 T cells share similar CD45RBlow, CD44high, LFA-1high, and CD62Llow phenotypes (13–15), although expression of CD45RB and CD62L may vary. CD45 isoform expression on effector cells appears to depend on activation conditions and cytokine environment (14, 16), whereas CD62L is expressed on a subset of memory CD4 T cells in unmanipulated mice (17). Expression of the IL-2R (CD25) is a characteristic of cycling cells and is often used to delineate effector from memory (18, 19). However, cycling is not necessarily required for effector cell function, as differentiated effectors in the form of T cell clones can produce cytokines such as IL-4 in the absence of proliferation (20).

Functionally, both effector T cells and activated memory T cells produce a similar array of effector cytokines, such as IFN-γ and IL-4, in addition to IL-2, while naive CD4 T cells produce primarily IL-2 (10, 11, 21). Effector and memory CD4 T cells also...
have similar requirements for activation relative to naive CD4 T cells, including reduced costimulation requirements (22).

We have taken a biochemical approach toward studying naive, effector, and memory CD4 T cell subsets. To date, we have identified striking differences in intracellular biochemical signaling events coupled to the TCR/CD3 complex in memory vs naive CD4 T cells isolated on the basis of CD45 isoform expression. We found that memory CD4 T cells exhibit anomalous signaling pathways when compared with naive or unfractonated CD4 T cells, which for the most part parallel signaling in T cell clones. Alterations in memory CD4 T cell signaling include differences in total tyrosine phosphorylation, a lack of the T cell-specific ZAP-70 kinase phosphorylation, and alterations in CD3ζ-associated proteins (23, 24). Our data indicate that biochemical analysis of CD4 T cell subsets may prove to be reliable criteria by which to assess their differentiation state.

Because generation and perpetuation of T cell memory can only occur in vivo, a number of adoptive transfer systems have been developed to follow the fate of Ag-specific effector CD4 or CD8 T cells obtained from TCR-transgenic mice in recipient mice depleted of T cells genetically (RAG–/–, CD8–/–) or by irradiation/thymectomy (19, 25–27). In these systems, T cells persisting after 2 wk to 3 mo that gave a recall response were deemed memory based on their longevity in vivo (18, 19, 26). All functional and phenotypic properties of these persisting cells were therefore ascribed to memory cells; however, whether true memory T cells were generated in these systems is unclear and cannot be assumed from the length of time that the transferred cells persist. Based on the finding of long-lived effector B cells (4), it is quite possible that the life span of effector T cells may be longer than previously thought. Furthermore, the phenotypic profile of the persisting T cells was not equivalent to that of resting memory cells isolated ex vivo (18, 19, 26). Thus, do the persisting T cells represent a long-lived effector population, a mixture of long-lived effector and resting memory, or an intermediate between effector and memory T cells? These issues have not yet been addressed.

In the present study we asked whether effector and memory CD4 T lymphocytes could be distinguished based on biochemical parameters, and whether these parameters could be used to follow the fate of effector cells in vivo. We analyzed total tyrosine phosphorylation in naive, effector, and memory CD4 T cells derived from unmanipulated mice and found that effector and memory subsets differ strikingly in the pattern and extent of total tyrosine phosphorylation. Biochemically, primary effectors derived from naive CD4 T cells are indistinguishable from secondary effectors derived from memory CD4 T cells. Using a combination of phenotypic, functional, and biochemical analyses, we compared an activated TCR-transgenic population that persisted in vivo following adoptive transfer into sublethally irradiated mice, to in vitro generated effector cells and ex vivo-isolated memory CD4 T cells. Based on these analyses, we found that the persisting activated population strongly resembles effector counterparts. We thus conclude that a subset of effector CD4 T cells can persist for several months in vivo without conversion to conventional memory CD4 T cells.

Materials and Methods

Mice

BALB/c mice were obtained from the National Cancer Institute Biological Testing Branch and were used between S and 12 wk of age. Breeding pairs of HA–TCR transgenic mice (28) were provided by Dr. Hyam Levitsky (Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD), bred as heterozygotes, and maintained in the Microbiology Animal Facility at the University of Maryland (College Park, MD). RHAββ (MHC class II+) mice (29) were purchased as a breeding pair from Taconic (Germantown, NY) and bred and maintained in the animal facility under sterile conditions.

Abs and reagents

The following Abs were purchased from culture supernatants from hybridomas provided by Dr. Kim Bottomly (Department of Immunobiology, Yale University Medical School, New Haven, CT) and maintained in the laboratory: C363.29B (anti-CD3ε) (30), GK1.5 (anti-CD8) (31), anti-CD8 (clone 3.56-67.2) (32), anti-CD4 (clone 2.43), and 212.A1 (anti-I-αI). The 6.5 anti-clonotype Ab (28) directed against the HA-TCR (rat IgG) hybridoma was provided by Dr. Hyam Levitsky (Johns Hopkins University School of Medicine), purified from culture supernatants, and conjugated to FITC (Pierce, Rockford, IL) according to the manufacturer’s recommendations. The following mAbs were purchased from Pharmingen (San Diego, CA): biotin-, FITC-, and PE-conjugated anti-CD45RB (clone C363.16A) (10), FITC- and PE-conjugated anti-CD44 (clone IM7); FITC- and PE-conjugated anti-IL-2R (CD25; clone 7D4); and PE-conjugated anti-CD4. The anti-phosphotyrosine mAb (clone 4G10) was purchased from Upstate Biotechnology (Lake Placid, NY).

The HA peptide 110–119 of the sequence SFERFEIFPK was synthesized on an Applied Biosystems 431a peptide synthesizer (Foster City, CA) using a PAL-PEG-PS resin. The peptide was analyzed by reverse phase HPLC.

Isolation of naive and memory CD4 cell subsets

The detailed procedure for isolation of mouse CD4 cells and subsequent sorting into CD45RBlow (memory) and CD45RBlow (naive) subsets was detailed previously (23). Briefly, CD4 cells were isolated from BALB/c spleen using immunomagnetic depletion with anti-CD8 and anti-I-αI mAbs followed by anti-CD4α, anti-CD4β, and anti-mouse IgM-coupled magnetic beads (PerSeptive Biosystems, Cambridge, MA). The resultant population was >90% CD4+ cells. CD4 T cells were fractionated into naive and memory subsets by positive and negative selection using MACS (Miltenyi Biotec, Sunnyvale, CA) separation. CD4 T cells were labeled with biotin-anti-CD45RB mAb (C363.16A, Pharmingen) followed by streptavidin MACS magnetic beads and subsequently separated into CD45RBlow and CD45RBhigh populations through a magnetic separation column fitted between a MACS magnet (Miltenyi Biotec). The resultant CD45RBlow (memory) and CD45RBhigh (naive) populations were >95% CD4.

In vitro generation of effector cells

T-depleted splenocytes as APC were prepared from BALB/c (MHC class IIα). or MHC class IIβ mice as previously described (23), using anti-Thy-1 (TIB238), anti-CD8 (TIB105), and anti-CD4 (GK1.5) plus rabbit complement (Accurate Chemical & Scientific, Westbury, NY). APC were treated with mitomycin C (Boehringer Mannheim, Indianapolis, IN) before use.

Primary effectors were generated by incubating the CD45RBlow subset with 10 μg/ml soluble anti-CD3 Ab (C363.29B) and MHC class II′ APC for 3 days at 37°C in complete Clicks medium consisting of Clicks (Irvine Scientific, Irvine, CA), 5% FCS (Bioproducts, Felsenthal, CA), 50 U/ml penicillin/streptomycin (Life Technologies/BRL, Grand Island, NY), 2 mM glutamine (Life Technologies), 10 mM HEPES (Life Technologies), and 50 μg/ml L-glutamine (Life Technologies). Effectors were purified through Ficoll, washed, and rested in complete Clicks medium for 24 h. Memory effectors were generated as above using CD45RBhigh subset incubated at 37°C for 3 days with anti-CD3 Ab and MHC class II′ APCs. For generation of Ag-activated effectors from HA-TCR mice, CD4 transgenic T cells (106 cells/ml) were cultured with APC (3 × 105 cells/ml) plus HA peptide (10 μg/ml) at 37°C in 24-well plates for 3 days.

Tyrosine phosphorylation analysis

The cells for biochemical analysis were activated and lysed as previously described (24). Briefly, cells at 2 × 106 cells in 100 μl of RPMI were activated for 2 min at 37°C with anti-CD3 Ab (C363.29B) plus goat anti-rat F(ab′)2 cross-linker (ICN Biomedical, Aurora, OH). Cells were lysed in cold 1% Nonidet P-40 lysis buffer with protease/phosphatase inhibitors as described previously (24). The lysates were analyzed by SDS-PAGE, gels were transferred to nitrocellulose, and blots were hybridized to anti-phosphotyrosine Ab as previously described (24). Bands were detected
using enhanced chemiluminescence (ECL, Amersham, Arlington Heights, IL) and were revealed with Hyperfilm ECL (Amersham).

**FACS analysis**

For staining, cells were washed and resuspended, and Abs were diluted in stain buffer (PBS, 2% FCS, and 0.05% sodium azide). Stained cells were analyzed using the FACScalibur (Becton Dickinson, San Jose, CA) with CellQuest software.

**Adaptive transfer of HA-TCR CD4 T cells**

For adoptive transfers, BALB/c mice were sublethally irradiated with 450 rad using a 60Co Cobalt source (Department of Nuclear Engineering, University of Maryland) 24 h before transfer of cells. For effector cell transfers, 3-day effectors were purified through Ficoll using LSM (ICN/Cappel, Warrington, PA), washed three or four times in PBS and resuspended in 10^5 to 2 x 10^5 cells/0.5 ml of PBS. For transfer of naive HA-TCR CD4 T cells, CD4 T cells were purified from naive HA-TCR mice and resuspended in PBS. Cells (0.5 ml) were transferred into sublethally irradiated BALB/c mice by injection into the tail vein. Controls received either PBS alone or equivalent numbers of BALB/c CD4 T cells. Adoptive transfer recipients were maintained in sterile conditions. Spleens were removed from recipient hosts at different time points (6–14 wk), and CD4 T cells were isolated as described above and assayed phenotypically, functionally, and biochemically. The majority of clonotype^+^ CD4 T cells were found in the spleen, and only negligible numbers were found in the peripheral lymph nodes.

**Proliferation and cytokine assays**

Ag-specific proliferation assays were set up in flat-bottom 96-well plates containing 50,000 purified CD4 T cells and 150,000 APC/well in complete Clicks medium plus titrated amounts of HA peptide. Proliferation was assessed after incubation at 37°C for 72 h by the addition of 1 μCi [3H]methylthymidine (6.7 Ci/mmol)/well, and cells were harvested 18 h later using a Tomtec 96-well plate harvester (Wallac, Gaithersburg, MD). Radioactivity was quantitated using a Microbeta Tri-luxe plate scintillation counter (Wallac). Supernatants from duplicate cultures set up for proliferation were collected after incubation at 37°C for 72 h by the addition of 1 μCi [3H]methylthymidine (6.7 Ci/mmol)/well, and cells were harvested 18 h later using a Tomtec 96-well plate harvester (Wallac, Gaithersburg, MD). Radioactivity was quantitated using a Microbeta Tri-luxe plate scintillation counter (Wallac). Supernatants from duplicate cultures set up for proliferation were collected after incubation at 37°C for 72 h by the addition of 1 μCi [3H]methylthymidine (6.7 Ci/mmol)/well, and cells were harvested 18 h later using a Tomtec 96-well plate harvester (Wallac, Gaithersburg, MD). Radioactivity was quantitated using a Microbeta Tri-luxe plate scintillation counter (Wallac). Supernatants from duplicate cultures set up for proliferation were collected after incubation at 37°C for 72 h by the addition of 1 μCi [3H]methylthymidine (6.7 Ci/mmol)/well, and cells were harvested 18 h later using a Tomtec 96-well plate harvester (Wallac, Gaithersburg, MD). Radioactivity was quantitated using a Microbeta Tri-luxe plate scintillation counter (Wallac).

**Results**

**Differential phosphorylation in naive, effector, and memory CD4 T cells**

Effector CD4 T cells have been shown to resemble long-lived memory counterparts both phenotypically and functionally. Because we had previously demonstrated striking differences in signaling coupled to the TCR/CD3 complex in mouse naive and memory CD4 T cells (23, 24), we asked whether effectors could likewise be biochemically distinguished from the memory subset. We initially investigated total tyrosine phosphorylation, which occurs immediately after triggering through the TCR/CD3 complex by Ag/MHC ligand or receptor cross-linking via anti-CD3 Abs. As a source of naive and memory CD4 T cells, we used CD45RB^high^ and CD45RB^low^ subsets of splenic CD4 T cells, previously shown to exhibit all of the phenotypic and functional attributes of naive and memory CD4 T cells, respectively (10, 11). Primary effector cells were generated in vitro from anti-CD3-mediated activation of naive (CD45RB^high^) CD4 T cells (see Materials and Methods).

An anti-phosphotyrosine immunoblot of cell lysates derived from resting and anti-CD3-stimulated naive (CD45RB^high^), primary effector, and memory (CD45RB^low^) CD4 T cells is shown in Fig. 1. Overall, there are striking differences in tyrosine phosphorylation among these three subsets, with effector CD4 T cells exhibiting increased tyrosine phosphorylation compared with equivalent numbers of memory or naive CD4 T cells, particularly in the absence of CD3 cross-linking (Fig. 1, compare lane eff/"0" to naive/"0" and mem/"0"). Following CD3 cross-linking, there is increased phosphorylation in all three subsets, although effector cells maintain the highest overall level of phosphorylation.

**FIGURE 1.** Tyrosine phosphorylation of naive, effector, and memory CD4 T cells. Anti-phosphotyrosine immunoblot of a 12% SDS-PAGE gel containing cell lysates derived from 10^6 cell equivalents of untreated or CD3-cross-linked naive, effector, and memory CD4 T cells. CD4 T cells isolated by immunomagnetic depletion were fractionated into naive (CD45RB^high^) and memory (CD45RB^low^) subsets (see Materials and Methods). Effector cells were generated from naive CD4 T cells by incubation for 3 days with soluble anti-CD3 and mitomycin C-treated T-depleted splenocytes, centrifuged over Ficoll to remove dead cells and APC, and incubated in fresh medium for 24 h before activation and lysis. Effector-specific (eff. sp.) and memory-specific (mem sp.) phosphorylated bands are indicated by arrows. Numbers to the left indicate m.w. standards. This blot is representative of six separate experiments.

In addition to these quantitative differences, there are effector-specific tyrosine phosphorylated proteins of 68 and 42–44 kDa (Fig. 1, see arrows) and those that are more highly phosphorylated in effector CD4 T cells, including species of 105–120, 76, and multiple (3–4) phosphorylated bands of 32–38 kDa compared with a broad band of 36 kDa in memory CD4 T cells lysates. These multiple phosphorylated species in the 36 kDa range may correspond to isoforms of the T cell linker-adaptor protein p36LAT (33). The effector-specific species exhibit increased phosphorylation in response to CD3 cross-linking, establishing that their phosphorylation is coupled to TCR signaling. Memory CD4 T cells exhibit memory-specific phosphorylated species at 28 and 12 kDa (Fig. 1, see arrow at right) not found in the effector subset. Thus, effector and memory CD4 T cells can be distinguished biochemically by both quantitative and qualitative differences in tyrosine phosphorylation.

**Biochemical analysis of primary and memory effectors**

The distinct biochemical profile of primary CD4 effector T cells compared with that of memory CD4 T cells suggests that different qualitative signals were being transduced through the TCR/CD3 complex in these two subsets. These differences may also indicate that effector and memory cells represent alternate differentiation states. To determine whether effector cells derived from the memory subset (memory effectors) exhibited memory-specific or effector-specific biochemical profiles, we compared the pattern of tyrosine phosphorylation of lysates derived from memory effectors to that of lysates from primary effectors. To generate memory effectors, we activated purified memory (CD45RB^low^) CD4 T cells with anti-CD3 and MHC class II^+^ splenic APC, as it has previously been shown that memory cells are not activated by anti-CD3.
presented by MHC class II$^+$ APC due to CD4-mediated negative signaling (23). Primary effectors were generated by activation of naive (CD45RB$^{high}$) CD4 T cells with anti-CD3 plus either MHC class II$^+$ or class II$^-$ APC. Shown in Fig. 2 is an anti-phosphotyrosine immunoblot of lysates derived from naive, primary effector, and memory effector CD4 T cells separated on an 8% gel to resolve the regions of disparity more closely. Here, the pattern of tyrosine phosphorylation in primary effectors is indistinguishable from the pattern seen in memory effectors (compare lanes 9 and 10 to lanes 3–6). The same highly phosphorylated, effector-specific bands of 32–38, 76, and 100–120 kDa were present in lysates derived from both types of effectors. The patterns of tyrosine phosphorylation of primary effectors generated by activation with MHC class II$^+$ and class II$^-$ APC were likewise identical (Fig. 2, lanes 3–6).

**Phenotypic and functional comparison of naive, memory and effector subsets**

Biochemical analysis of naive T cells, memory T cells, and the effectors derived from these subsets suggested that primary and memory effector CD4 T cells exist in similar differentiation states, distinct from the memory subset. To assess the differentiation state of the effector subsets further, we analyzed their phenotypic and functional profile compared with those of naive and memory counterparts. Shown in Fig. 3A is the expression of activation/memory markers CD45RB, CD44, CD62L, and IL-2R (CD25) on sorted naive and memory CD4 T cells from spleen, and primary and secondary effectors derived from these subsets in vitro. Naive (CD45RB$^{high}$) CD4 T cells isolated ex vivo were CD44$^{low}$, IL-2R$^{low}$, and heterogeneous for CD62L expression, whereas memory CD4 (CD45RB$^{low}$) T cells were CD44$^{high}$, CD62L$^{low}$, and IL-2R$^{low}$ (Fig. 3A, columns 1 and 3) as has been seen previously (34). Both naive and memory CD4 T cells were also small in size (Fig. 3A, row 5, columns 1 and 3), demonstrating that these subsets represent small, resting lymphocytes.

**FIGURE 2.** Primary vs secondary effectors. A. A phosphotyrosine immunoblot of an 8% SDS-PAGE gel containing lysates derived from $10^6$ fractionated naive (CD45RB$^{high}$, lanes 1 and 2), memory (CD45RB$^{low}$; lanes 7 and 8), primary effectors generated from activation of naive cells with either anti-CD3 and BALB/c splenic APC (lanes 3 and 4) or anti-CD3 and MHC class II$^+$ APC (lanes 5 and 6), memory effectors generated by activation of CD45RB$^{low}$ cells with anti-CD3 and MHC class II$^-$ APC (lanes 9 and 10). Arrows to the right of the gel point to effector-specific bands. This blot is representative of three separate experiments.

**FIGURE 3.** Phenotypic and functional analysis of primary vs secondary effectors. Naive and memory CD4 T cells subsets were isolated, and effectors were generated from them as described in Fig. 2. A. Cells were double stained for CD4 and CD45RB, CD44, CD62L, or IL-2R. Histograms represent staining profiles or FSC gated on the CD4$^+$ fraction. This figure is representative of three separate experiments. B. Analysis of effector cytokine expression in CD4 T cells subsets activated by anti-CD3-mediated cross-linking after 48 h. Bars marked no Ab indicate cells cultured alone, and bars marked + CD3 indicate cells cultured on immobilized anti-CD3 Ab. Value labels are indicated for each histogram. C. Effector cytokine production in response to Ag/APC by Ag-activated effectors derived by activation of HA-TCR CD4 T cells with HA peptide and APC for 3 days. Effectors were Ficoll purified, washed, and recultured alone (no APC), with BALB/c splenic APC (+ APC), or with HA peptide and APC. Supernatants were collected 48 h later, and IFN-$\gamma$ content was quantitated by ELISA.

Effectors CD4 T cells generated from either naive or memory CD4 T cells by activation with anti-CD3 and MHC class II$^+$ or MHC class II$^-$ APC, respectively, exhibited a profound increase in size (Fig. 3A, row 5, columns 2 and 4), indicative of blast-like, cycling cells, and exhibited up-regulation of CD44 and IL-2R expression (Fig. 3A, columns 2 and 4). (Primary effectors generated from naive CD4 T cells activated with anti-CD3 and either MHC class II$^+$ or II$^-$ APC exhibited similar phenotypes (data not shown)) Despite the similarities in CD44 and IL-2R expression, the pattern of CD45RB and CD62L expression on the surface of primary vs memory effector cells differed. CD45RB expression remained high on primary effectors and was partially up-regulated in memory effectors, whereas CD62L expression was heterogeneous on primary effectors and remained low on memory effectors. A similar heterogeneity in CD45RB and CD62L expression was recently demonstrated in Ag-activated effectors derived from TCR-transgenic CD4 T cells (35). Our phenotype results indicate that effector and memory CD4 T cells can be distinguished based
on up-regulation of CD45RB and CD25 expression and heterogeneity of CD62L expression. However, the degree of CD45RB up-regulation and CD62L heterogeneity depends on the identity of the resting precursor.

We also assessed the ability of naive, effector, and memory subsets to produce the effector cytokine, IFN-γ, in response to TCR/CD3 cross-linking. Because TCR/CD3 cross-linking yielded disparate tyrosine phosphorylation profiles among naive, effector, and memory subsets (Fig. 2), we wished to determine whether these signaling differences could translate into disparities in downstream events. We cultured freshly isolated naive and memory CD4 T cells subsets and primary and memory effectors derived in vitro with plastic-immobilized anti-CD3 Abs and analyzed the IFN-γ content in supernatants after 48 h. As shown in Fig. 3B, while naive CD4 T cells, as expected (10, 11), did not produce IFN-γ, primary effector, memory, and memory effector CD4 T cells produced high levels of IFN-γ in response to TCR/CD3 cross-linking, although the level of IFN-γ production by effector subsets was twice that seen with activated memory CD4 T cells. Effectors derived from either naive or memory CD4 T cells did not exhibit different levels or kinetics of cytokine production (Fig. 3B and data not shown), further indicating that primary and memory effectors do not differ substantially in terms of signaling or functional outcome. Interestingly, neither effector population produced significant levels of IFN-γ in the absence of TCR/CD3 stimulation, indicating that when taken out of the activating stimulus, at least a subset of effector cells can be restimulated. These data demonstrate that effector and memory cells are functionally similar in terms of downstream events, such as cytokine production.

To establish whether effector CD4 T cells can likewise be restimulated to produce effector cytokines in response to Ag activation, we generated Ag-specific effector cells in vitro by activation of CD4 T cells derived from TCR-transgenic mice. We used HA-TCR-transgenic mice on BALB/c genetic backgrounds containing CD4 T cells derived from TCR-transgenic mice. We used HA-TCR mice. We generated Ag-activated HA-TCR effector cells in vitro as described above and subsequently transferred them into sublethally irradiated syngeneic BALB/c mice. When compared with previous transfers into RAG2−/− or lethally irradiated empty hosts, sublethal irradiation initially creates space for the transferred effector cells, yet allows for the eventual repopulation of endogenous T cells. At various time points following transfer, splenic CD4 T cells were isolated from adoptive transfer recipient mice and analyzed phenotypically, functionally, and biochemically.

The cell surface phenotypes and sizes of naive HA-activated effectors and persisting HA-TCR cells 6 or 10 wk post-transfer are shown in Fig. 4A, defined by positive staining with the 6.5 anti-clonotype Abs. Clonotype (6.5+) CD4 T cells isolated from HA-TCR mice (naive) are primarily CD45RBhigh CD62Lhigh IL-2Rlow. CD4 cells, express heterogeneous levels of CD44; and are small in size, with a mean FSC of 395 (Fig. 4, column 1). Effector cells isolated following activation of these same naive HA-TCR CD4 T cells with HA peptide plus BALB/c splenic APC, or with HA peptide/APC, and data not shown, indicate that primary and memory effectors do not differ substantially in terms of signaling or functional outcome. Interestingly, neither effector population produced significant levels of IFN-γ in the absence of TCR/CD3 stimulation, indicating that when taken out of the activating stimulus, at least a subset of effector cells can be restimulated. These data demonstrate that effector and memory cells are functionally similar in terms of downstream events, such as cytokine production.

To analyze the functional properties of these persisting cells, we transferred relatively high numbers (107 to 2 × 107) of effector cells, analyzed the cell surface phenotype of the endogenous T cells isolated from these same recipient mice. For all surface markers, CD4, CD44, CD45RB, CD62L, and IL-2R, the pattern of expression of nonclonotype CD4 T cells was equivalent in mice that had received PBS or effector cells (Fig. 4B and data not shown). For comparison, the expression of CD44 and IL-2R on the surface of endogenous CD4 T cells is shown in Fig. 4B. These results demonstrate that there are no overt bystander effects of the transferred effectors on the endogenous CD4 T cell population.

To analyze the functional properties of these persisting cells, we assayed purified CD4 T cells from these same adoptive transfer recipient mice for their ability to respond to HA peptide. As shown in Fig. 5, the CD4 T cells persisting 6, 10, or 14 wk post-transfer...
respond more vigorously and produce higher levels of cytokines than naive HA-TCR counterparts. Fig. 5A shows the dose response of Ag-specific proliferation of fresh naive HA-TCR transgenic CD4 T cells compared with that of equivalent numbers of CD4 T cells derived from mice that received either effector cells or PBS 6 or 10 wk previously. (The proportion of 6.5+ CD4 T cells in both naive and transfer recipients was 30–35%.) While fresh naive HA-TCR CD4 T cells responded in a dose-dependent fashion, peaking at 10 μg/ml of peptide, CD4 T cells derived from mice that received effectors responded maximally to much lower doses of peptide, specifically 0.5–1 μg/ml of peptide Ag. Similarly, CD4 T cells persisting 14 wk after transfer of effector cells responded at much lower doses compared with equivalent numbers of naive CD4 T cells that persisted 14 wk post-transfer (Fig. 5B).

The proliferation results from Fig. 5, A and B, are normalized for equivalent numbers of 6.5+ CD4 T cells and presented in Fig. 5C. As shown in this graph, at least a subset of the transferred effector cells continue to turnover in vivo, as exemplified by proliferation in the absence of Ag. This background proliferation decreases with increased time. Ag-specific proliferation in response to a low dose (0.5 μg/ml) is 20- to 40-fold higher in 6.5+ transferred effector cells than in equal numbers of fresh naive 6.5+ cells or adoptively transferred naive 6.5+ cells. Proliferation of transferred effectors peaked 10 wk post-transfer and decreased with time in adoptive hosts.

Although Ag-specific proliferation varied with time in adoptive hosts, production of IFN-γ in response to Ag remained highly elevated in mice that had received effectors 6, 10, or 14 wk previously (Fig. 5D). This response (normalized for equivalent numbers of 6.5+ CD4 T cells) greatly surpassed IFN-γ production by fresh naive HA-TCR CD4 T cells or naive HA-TCR CD4 T cells that persisted in adoptive hosts. Thus, the HA-TCR T cells persisting following transfer of effector cells mediate a recall response as exemplified by their ability to respond more vigorously to lower doses of Ag and produce greatly elevated levels of effector cytokines compared with naive counterparts.

**Biochemical analysis of persisting CD4 T cells**

Because the persisting HA-TCR T cells exhibited functional attributes of memory CD4 T cells, yet phenotypically exhibited characteristics of both effector and memory CD4 T cells, we asked whether biochemical analysis could resolve these discrepancies. Thus, we analyzed the pattern of total tyrosine phosphorylation in CD4 T cells isolated from individual mice that had received PBS alone, BALB/c CD4 T cells, naive HA-TCR T cells, or effector cells (Fig. 6). CD4 T cells isolated from mice that received unstimulated CD4 T cells or PBS exhibited a naive-like pattern of tyrosine phosphorylation, with low levels of phosphorylation in the resting state and induction of phosphorylation at 36 kDa, 70–90 kDa, and 100–130 kDa following CD3 cross-linking (Fig. 6, lanes 3–8). However, CD4 T cells isolated from mice that had received effector cells either 6 or 10 wk previously exhibited a profound effector-like biochemical profile (Fig. 6, lanes 9–14). (At 14 wk, the proportion of 6.5+ cells was too low to yield definitive biochemical results.) This effector-like profile included a higher overall level of tyrosine phosphorylation compared with CD4 T cells isolated from mice that received unstimulated CD4 T cells and the appearance of effector-specific phosphorylated species of 42–44 kDa, multiple bands at 36–38 kDa, a band at 76 kDa, and strong phosphorylation at 100–130 kDa (Fig. 6, lanes 9–14). No memory-specific phosphorylated species of 28 and 12 kDa were observed in the tyrosine phosphorylation profile of CD4 T cells derived from mice that received effector cells (Fig. 6 and data not shown). These results suggest that the predominant persisting population following adoptive transfer of activated effector cells biochemically resembles effector cells.
Discussion

We have taken a biochemical approach toward analyzing T cell memory and its generation in vivo by examining the intracellular tyrosine phosphorylation events of effector and memory CD4 T cell subsets and activated CD4 T cells that persist in vivo. As presented here, we found that effector and memory CD4 T lymphocytes could be clearly distinguished by quantitative and qualitative differences in tyrosine phosphorylation. We used biochemical criteria in addition to phenotypic and functional analyses to follow the fate of in vitro generated, Ag-specific CD4 effector T cells transferred into adoptive hosts, to give a more complete picture of the nature of the persisting cell population. The transferred cells persisted in vivo for at least 3.5 mo, mediated a recall response when stimulated with cognate Ag, and both proliferation and production of the cytokine IFN-γ were assessed. A, Ag-specific dose response of CD4 T cells isolated from naive HA-TCR mice vs individual mice that had received HA-TCR effectors 6 or 10 wk previously. The results from mice that had received PBS 6 or 10 wk previously are identical and are marked PBS. The 6.5+ population in both naive mice and mice that had received effectors was 31–36% of the total CD4 T cells. B, Ag-specific dose response of CD4 T cells isolated from mice that had received either 10⁷ naive HA-TCR or 10⁷ effector HA-TCR 14 wk previously. The percentage of persisting 6.5+ cells is 15% for both effector and naive transfer recipient mice. C. Normalized proliferative response from A and B, comparing Ag-specific proliferation for equal numbers of 6.5+ CD4 T cells. D. Normalized production of IFN-γ from equal numbers of 6.5+ CD4 T cells derived from mice that had received PBS or 10⁷ effector cells 6, 10, or 14 wk previously or naive HA-TCR CD4 T cells 14 wk previously compared with the production of IFN-γ from freshly isolated naive HA-TCR CD4 T cells. The Ag concentration is 0.5 μg/ml.

FIGURE 5. Functional analysis of persisting HA-TCR CD4 T cells. Splenic CD4 T cells were isolated from mice that had received HA-TCR effector cells 6, 10, or 14 wk previously, naive CD4 T cells 14 wk previously, or PBS and were stimulated in vitro with different doses of peptide Ag, and both proliferation and production of the cytokine IFN-γ were assessed. A, Ag-specific dose response of CD4 T cells isolated from naive HA-TCR mice vs individual mice that had received HA-TCR effectors 6 or 10 wk previously. The results from mice that had received PBS 6 or 10 wk previously are identical and are marked PBS. The 6.5+ population in both naive mice and mice that had received effectors was 31–36% of the total CD4 T cells. B, Ag-specific dose response of CD4 T cells isolated from mice that had received either 10⁷ naive HA-TCR or 10⁷ effector HA-TCR 14 wk previously. The percentage of persisting 6.5+ cells is 15% for both effector and naive transfer recipient mice. C. Normalized proliferative response from A and B, comparing Ag-specific proliferation for equal numbers of 6.5+ CD4 T cells. D. Normalized production of IFN-γ from equal numbers of 6.5+ CD4 T cells derived from mice that had received PBS or 10⁷ effector cells 6, 10, or 14 wk previously or naive HA-TCR CD4 T cells 14 wk previously compared with the production of IFN-γ from freshly isolated naive HA-TCR CD4 T cells. The Ag concentration is 0.5 μg/ml.

Effector vs memory vs persisting effector CD4 T cells

We have analyzed effector CD4 T cells, CD4 T cells that persisted in vivo following transfer of effector cells, and memory CD4 T cells isolated ex vivo from unmanipulated mice based on three criteria: biochemical profile, phenotype, and function. Our results are summarized in Table I. Biochemically, effector and memory subsets exhibit quantitative and qualitative differences in the pattern of tyrosine phosphorylation, with effector cells exhibiting high

rightly from short-lived, activated effector T cells that have reverted to the resting state. We believe that the results presented here call into question two assumptions inherent in the statement above. First, our data challenge previous assertions that effector T cells are short-lived, because previously activated CD4 T cells persisting in vivo, biochemically resembled effector and not memory CD4 T cells. Second, our finding of persisting effector cells coupled with biochemical analysis of primary effector, memory, and memory effector CD4 T cells also calls into question whether memory T cells represent differentiated effector cells that have reverted to the resting state.
The biochemical profile of the persisting cells matches an effector-like phenotype. However, phenotype has not previously been shown to reliably distinguish effector and memory CD4 T lymphocytes, the persisting 6.5+ T cells exhibit certain phenotypic characteristics of primary effector cells (see Table I). First, the persisting population exhibited a predominant CD45RBhigh phenotype (with some limited heterogeneity), similar to the primary effectors we generated in vitro with either Ag or anti-CD3, whereas conventional ex vivo memory CD4 T cells are typically CD45RBlow (11, 41). Second, the persisting cells exhibited heterogeneous CD62L expression similar to primary effectors (Figs. 3 and 4). By contrast, ex vivo memory CD4 (CD45RB) T cells are typically CD62Llow (see Fig. 3). It is not known whether CD62L heterogeneity reflects a mixture of persisting memory and effector cells or an intermediate between effector and memory T cells. Interestingly, persisting activated CD4 and CD8 T cells in other adoptive transfer systems were also shown to be heterogeneous for CD62L expression (18, 38, 42), suggesting that the persisting population following transfer of effector cells is itself heterogeneous. We propose that biochemical analysis of phenotypic subsets of effector/memory T cells may enable a more precise assignment of phenotypic markers to the appropriate differentiation state. Sorting of these mixed phenotypes may also reveal whether a mixture of long-lived effectors and conventional memory T cells persists in the adoptive hosts.

While the persisting 6.5+ CD4 T cells exhibited primary effector-like surface marker expression, they exhibited two phenotypic properties normally associated with resting cells: smaller size and loss of IL-2R expression. The 6.5+ cells persisting after transfer of blast-like effector cells were smaller in size than effectors, yet not quite as small as resting naive CD4 T cells (see Fig. 4) or memory CD4 T cells.

The biochemical profile of the persisting cells matches an effector-specific profile (see Table I), indicating that a predominant population of transferred effectors remains effector cells and does not revert to resting memory cells. This biochemical profile was clearly visible in CD4 T cells isolated from adoptive transfer recipients where 35% of the persisting cells were derived from the transferred population. Although we did not detect the p28 or p12 phosphorylated species that unequivocally distinguish these two subsets. Some of the effector-specific bands correspond to the sizes of known signaling intermediates; for example, multiple bands at 34–38 kDa may correspond to differentially phosphorylated isoforms of p36LAT (33), and effector bands of 42–46 kDa may correspond to highly phosphorylated mitogen-activated protein kinase species. Furthermore, phosphorylated bands of 100–130 kDa may correspond to linker/adapter signaling proteins such as Vav, Fyb/SLAP, and/or p120cbl (40). We are beginning to characterize the phosphorylation state of these specific signaling intermediates in effector vs memory CD4 T cells with the goal of identifying markers that unequivocally distinguish these two subsets.

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Although phenotype has not previously been shown to reliably distinguish effector and memory CD4 T lymphocytes, the persisting 6.5+ T cells exhibit certain phenotypic characteristics of primary effector cells (see Table I). First, the persisting population exhibited a predominant CD45RBhigh phenotype (with some limited heterogeneity), similar to the primary effectors we generated in vitro with either Ag or anti-CD3, whereas conventional ex vivo memory CD4 T cells are typically CD45RBlow (11, 41). Second, the persisting cells exhibited heterogeneous CD62L expression similar to primary effectors (Figs. 3 and 4). By contrast, ex vivo memory CD4 (CD45RB) T cells are typically CD62Llow (see Fig. 3). It is not known whether CD62L heterogeneity reflects a mixture of persisting memory and effector cells or an intermediate between effector and memory T cells. Interestingly, persisting activated CD4 and CD8 T cells in other adoptive transfer systems were also shown to be heterogeneous for CD62L expression (18, 38, 42), suggesting that the persisting population following transfer of effector cells is itself heterogeneous. We propose that biochemical analysis of phenotypic subsets of activated/effector/memory T cells may enable a more precise assignment of phenotypic markers to the appropriate differentiation state. Sorting of these mixed phenotypes may also reveal whether a mixture of long-lived effectors and conventional memory T cells persists in the adoptive hosts.

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**Table I. Summary of biochemical, phenotypic, and functional characteristics of effector CD4 T cells following adoptive transfer of effector cells (“Persisting Effector”), and memory CD4 T cells isolated from unmanipulated mice**

<table>
<thead>
<tr>
<th>Effector</th>
<th>“Persisting Effector”</th>
<th>Memory</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biochemical profile</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>High level of Tyr-P, p42–46 phosphorylated</td>
<td>Low level of Tyr-P, p28 phosphorylated</td>
</tr>
<tr>
<td><strong>Phenotype</strong></td>
<td>CD45RB&lt;sup&gt;high&lt;/sup&gt;</td>
<td>CD45RB&lt;sup&gt;high/low&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CD4&lt;sup&gt;bright&lt;/sup&gt;</td>
<td>CD4&lt;sup&gt;bright&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>CD62L&lt;sup&gt;mixed&lt;/sup&gt;</td>
<td>CD62L&lt;sup&gt;low/high&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>IL-2R&lt;sup&gt;high&lt;/sup&gt;</td>
<td>IL-2R&lt;sup&gt;low&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Size</strong></td>
<td>Large</td>
<td>Smaller/intermediate</td>
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<tr>
<td><strong>Cytokine Production</strong></td>
<td>Effector cytokines</td>
<td>Effector cytokines</td>
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</tbody>
</table>

**FIGURE 6.** Biochemical analysis of CD4 T cells from adoptive transfer recipients. Lysates derived from equivalent numbers (10<sup>6</sup>) of fresh effector CD4 T cells (lanes 1 and 2) or CD4 T cells purified from individual mice that had received PBS (lanes 3 and 4), 10<sup>5</sup> BALB/c CD4 T cells (lanes 5 and 6), 10<sup>5</sup> naive HA-TCR T cells (lanes 7 and 8), 10<sup>5</sup> effector cells 6 wk (lanes 9 and 10) or 10 wk (lanes 13 and 14) previously, or 2 × 10<sup>5</sup> effectors 6 wk previously (lanes 11 and 12) were resolved on a 12% polyacrylamide gel, blotted to nitrocellulose, and hybridized with anti-phosphotyrosine. Arrows to the right of the blot designate effector-specific bands.

Biochemical analysis of CD4 T cells from adoptive transfer recipients. Lysates derived from equivalent numbers (10<sup>6</sup>) of fresh effector CD4 T cells (lanes 1 and 2) or CD4 T cells purified from individual mice that had received PBS (lanes 3 and 4), 10<sup>5</sup> BALB/c CD4 T cells (lanes 5 and 6), 10<sup>5</sup> naive HA-TCR T cells (lanes 7 and 8), 10<sup>5</sup> effector cells 6 wk (lanes 9 and 10) or 10 wk (lanes 13 and 14) previously, or 2 × 10<sup>5</sup> effectors 6 wk previously (lanes 11 and 12) were resolved on a 12% polyacrylamide gel, blotted to nitrocellulose, and hybridized with anti-phosphotyrosine. Arrows to the right of the blot designate effector-specific bands.
(CD45RB<sup>low</sup>) CD4 T cells (Figs. 3 and 4). Persisting cells in different mice exhibited consistent and reproducible mean FSC of 428–450 up to 22 wk in adoptive hosts (data not shown), whereas resting naive and memory subsets exhibited mean FSC of 395–397. When taken together with our biochemical and phenotype results presented here, the fact that the persisting cells do not exactly revert to the small state found in vivo in unmanipulated mice suggests that while a reduction in size is generally associated with differentiation to memory T cells, this phenomenon may also occur to varying extents in long-lived noncycling effector T cells.

The majority of 6.5<sup>+</sup> CD4 T cells persisting after adoptive transfer of effector cells down-regulate expression of the IL-2R, although there is a small subset that expresses low levels of this receptor, consistent with the findings of Swain (19). Loss of IL-2R expression has likewise been used as evidence of conversion to memory in adoptive transfers of activated CD4 (18, 19) and CD8 T cells (27). However, loss of IL-2R expression has been found in vivo on activated cells that have not converted to resting memory T cells. Effector-type CD4 T cells in humans expressing the markers CD45RO, CD69, and HLA-DR characteristic of recently activated cells (43), yet lacking CD25 (IL-2R) expression, have been found in the synovium of rheumatoid arthritis patients (44) and among tumor-infiltrating lymphocytes in renal carcinoma (45). These findings suggest that in vivo, IL-2R expression may be down-regulated during chronic or large scale activation and result in a noncycling effector cell that continues to secrete cytokines. Furthermore, recent findings showing that IL-2 promotes apoptosis of normal activated T cells (46) suggest that IL-2R down-regulation may be required for survival in the periphery and does not necessarily reflect a reversion to the resting state.

Despite the predominant down-regulation of IL-2R expression, at least a subset of the persisting effectors proliferates in vitro in the absence of Ag, which declines with time in adoptive hosts (Fig. 5). We have continued to observe this nonspecific proliferation of 6.5<sup>+</sup> CD4 T cells isolated from mice that received effector cells 22 wk (almost 6 mo) previously (data not shown). Our results are consistent with previous studies showing that a subset of activated CD8 T cells transferred into irradiated/CD8<sup>-/-</sup> recipient mice continue to cycle in vivo for up to 6 mo (27). This continuous cycling in the absence of IL-2R expression appears paradoxical; however, it has been shown that CD25<sup>-/-</sup> T cells can continue to cycle in response to other cytokines, such as IL-15 and IL-4 (47). The presence of both cycling and noncycling CD4 T cell populations expressing activated/memory phenotypes in vivo in unmanipulated mice (48) suggests that a continuously cycling, previously activated population is not an artifact of these adoptive transfer systems.

What is the basis for the continuous cycling of activated cells persisting in vivo? The effector cells were transferred into a BALB/c host in the absence of Ag, suggesting that continuous turnover of previously activated CD4 T cells does not require Ag, although we cannot rule out that minute amounts of HA peptide may have bound to the surface of the washed and purified effector cells before transfer. Alternately, these effector cells may continue to cycle based on cross-reactions to self or other environmental Ags, a mechanism that has been suggested for the maintenance of long-term immunological memory (27, 49).

Functionally, the persisting activated cells give a recall response, as exemplified by their ability to respond to low doses of Ag and produce highly elevated levels of cytokines compared with naive counterparts. We have shown here that our in vitro generated effector cells can also be restimulated and produce effector cytokines (see Fig. 3). It has been shown in other systems that effector cells generated in vivo 5–7 days after immunization with Ag give recall responses when stimulated in vitro (14); however, 30 days postimmunization, these same recall responses are believed to be exclusively mediated by memory T cells (5, 50). We hypothesize that anamnestic T cell responses occurring at least several months after initial Ag encounter could be mediated by a combination of persisting effector T cells and reactivated memory T cells.

**Generation of memory T cells**

If a predominant persisting population derived from activated effector cells is made up of effectors, then how are long-lived resting memory T cells generated? Although the persisting population exhibited qualities of effector cells when examined biochemically, functionally, and by certain phenotypic parameters (see Table 1), we cannot rule out that a mixed population of long-lived effector and memory subsets resulted, and detection of the memory population was masked by the dominant effector-specific biochemical profiles and ambiguous phenotypes. In contrast to the classical model of generation of memory T cells directly from activated effectors, we and others (51) favor a divergent model for memory T cell generation distinct from effector cells. We have proposed a differential signaling mechanism for the generation of memory T cells (52), whereby partial activation of a naive cell gives rise to a long-lived memory T cell, and full activation leads to effector cell generation. We base this mechanism on evidence that memory CD4 T cells biochemically and functionally resemble mature T cell clones that have been partially activated by altered peptide ligands exhibiting lower TCR affinities than wild-type antigenic peptides (23, 24, 53, 54). Currently, we are testing whether the mode of activation determines whether effector or memory cells predominate in adoptive hosts, including the Ag dose used to generate effectors, the number of effectors transferred, and the type and extent of costimulation.

Our findings that both primary and secondary effectors share similar biochemical profiles may provide insight into the relationship of effector to memory T cells. It is generally believed that naive CD4 T cells must differentiate to become effector cells, whereas memory T cells are already differentiated. Functionally, both memory and effector cells represent differentiated cells able to produce effector cytokines when activated by TCR cross-linking alone. However, if memory CD4 T cells arise from effector cells, then we would expect the biochemical profile of primary effectors to resemble that of CD3-stimulated memory cells. Yet this was not the case. We found that phosphorylation in primary effectors qualitatively differed from CD3-cross-linked memory CD4 T cells (Figs. 1 and 2), suggesting either that effector and memory T cells are generated by divergent pathways or that a differentiation event beyond a simple reversion to the resting state must occur for effectors to become memory CD4 T cells. Moreover, if memory CD4 T cells are already fully differentiated to become effectors, then the biochemical profile of CD3-cross-linked memory T cells should closely resemble the profile of secondary effectors, yet this also was not the case. This disparity suggests that additional differentiation must occur for a memory CD4 T cell to become an effector cell, and that memory CD4 T cells may exist in their own novel differentiation state, distinct from naive and effector T cells.

In Fig. 7, we present a model to explain the differentiative relationship of naive, primary effector, memory, and memory effector subsets. In this model, effector cells represent terminally differentiated subsets and are generated distinct from memory subset. Primary effector cells either die or persist in the effector state. Memory CD4 T cells, by contrast, persist unless activated to become an effector T cell. This model predicts that a memory recall response can be due to reactivation of at least two types of previously activated cells: persisting effector cells and differentiated
memory cells. Effector cells generated from the memory subset are either differentiated to memory effectors in a parallel fashion as primary effectors, or alternately, only one type of effector population exists, and memory CD4 T cells represent a long-lived intermediate between naive and effector T cells. Because it is difficult to biochemically and functionally distinguish between primary and secondary (memory) effectors, either of these scenarios is possible.

Opferman et al. (55) report that they have demonstrated linear differentiation of effector CD8 T cells to the memory subset. These investigators transferred in vitro activated TCR-transgenic CD8 T cells, sorted into pre-effector and posteffector states by cell cycle labeling, into RAG−/− hosts. Only the posteffector sorted population gave an increased CTL precursor frequency characteristic of recall responses. However, assignment of the persisting cells as memory T cells was based solely on longevity in vivo (10 wk). We conclude from our results presented here that examination of one parameter cannot reliably designate an activated or persisting activated cell as a memory cell. Rather, a combination of parameters, including biochemical analysis, is necessary to precisely determine the differentiation state of an activated T cell. Thus, the persisting cells in the study by Opferman may represent effector cells that have not undergone further differentiation into resting memory cells.

The results presented here demonstrate that analysis of signaling pathways can provide an unambiguous mechanistic assessment of the activation/differentiation state of CD4 T cells. Using biochemical analysis, we have shown that a subset of effector CD4 T cells can persist several months in vivo, contrasting with previous assertions that effector cells lasted only days to weeks before dying by apoptosis. Determining the mechanisms underlying the generation and persistence of long-lived effectors vs resting memory T cells will be invaluable for improved design of vaccines to generate an effective anamnestic immune response.

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