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*J Immunol* 2002; 168:1557-1565; doi: 10.4049/jimmunol.168.4.1557
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Differential SLP-76 Expression and TCR-Mediated Signaling in Effector and Memory CD4 T Cells

S. Farzana Hussain, Charles F. Anderson, and Donna L. Farber

We present in this study novel findings on TCR-mediated signaling in naive, effector, and memory CD4 T cells that identify critical biochemical markers to distinguish these subsets. We demonstrate that relative to naive CD4 T cells, memory CD4 T cells exhibit a profound decrease in expression of the linker/adapter molecule SLP-76, while effector T cells express normal to elevated levels of SLP-76. The reduced level of SLP-76 in memory CD4 T cells is coincident with reduced phosphorylation overall, yet the residual SLP-76 couples to a subset of TCR-associated linker molecules, leading to downstream mitogen-activated protein (MAP) kinase activation. By contrast, effector CD4 T cells strongly phosphorylate SLP-76, linker for activation of T cells, and additional Grb2-coupled proteins, exhibit increased associations of SLP-76 to phosphorylated linkers, and hyperphosphorylate downstream Erk1/2 MAP kinases. Our results suggest distinct coupling of signaling intermediates to the TCR in naive, effector, and memory CD4 T cells. Whereas effector CD4 T cells amplify existing TCR signaling events accounting for rapid effector responses, memory T cells engage fewer signaling intermediates to efficiently link TCR triggering directly to downstream MAP kinase activation. The Journal of Immunology, 2002, 168: 1557–1565.

Effector and memory T cells both constitute previously activated T cells with enhanced responses relative to naive T cell counterparts. Both have greatly reduced requirements for costimulation (1, 2), elicit potent effector function, share similar cell surface phenotypes (3), and acquire the potential to efficiently home to nonlymphoid tissue (4). Despite these similarities, effector and memory T cells have different activation properties and experience different fates in an immune response. Effector T cells are generally hyper-responsive to antigenic and nonantigenic stimuli (2, 5) and exhibit a high susceptibility to AICD (6), resulting in short term nonantigenic stimuli (2, 5) and exhibit a high susceptibility to AICD.

D.L.F.

Received for publication September 13, 2001. Accepted for publication November 30, 2001.

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1 This work was supported by National Institutes of Health Grant AI42092 (to D.L.F.).
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3 Abbreviations used in this paper: AICD, activation-induced cell death; HA, hemagglutinin; MAP, mitogen-activated protein; GADS, Grb2-related adapter downstream of Shc.

Materials and Methods

Mice

BALB/c mice, between 6 and 8 wk of age, were obtained from the National Cancer Institute Biological Testing Branch. Hemagglutinin (HA)-TCR transgenic mice expressing the TCR specific for influenza HA peptide and
Differential Signaling in Effector and Memory T Cells

I-E\(^d\) (21) and MHC class II\(^{\text{ID}}\) mice (Taconic Farms, Germantown, NY) were bred and maintained at the animal care facility of University of Maryland School of Medicine (Baltimore, MD).

Abs and reagents

The following Abs were purified from culture supernatants from hybridomas and maintained in the laboratory as described previously (20); anti-CD3e (C363.29B), anti-CD4 (GK1.5), anti-CD8 (TIB 105), anti-\(\text{I-A}^d\) (212.A1), and anti-Thy-1.2 (TIB 238). PE-conjugated anti-CD45RB (clone C363.16A), used for separation of CD45RB\(^{\text{HI}}\) and CD45RB\(^{\text{LO}}\) CD4 T cells, was obtained from BD Pharmingen (San Diego, CA). For Western blotting experiments, anti-phosphotyrosine (mouse IgG2b, clone 4G10), anti-ERK1/2, and anti-phospholipase C (PLC\(\gamma\)) (clone P-4, Santa Cruz Biotechnology, Santa Cruz, CA) Abs were purchased from Upstate Biotechnology (Lake Placid, NY), anti-phospho-Erk2 Ab (mouse IgG2a) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-SLP-76 (Ab 22) was generously provided by Dr. G. A. Koretzky (University of Pennsylvania, Philadelphia, PA), anti-LAT antiserum (12) was kindly provided by Dr. R. Wange (National Institutes of Aging, Baltimore, MD), and anti-GADS antiserum (23) was kindly provided by Dr. C. J. McGlade (Hospital for Sick Children, Toronto, Ontario, Canada). For the Grb2-GST pull-down experiments, a GST-Grb2 fusion protein coupled to agarose (Upstate Biotechnology) and GST-agarose (Pierce, Rockford, IL) were used. The HA peptide 110–119 of the sequence, SFERFEIPFPK, was synthesized by the Biopolymer Laboratory, University of Maryland School of Medicine.

Isolation of naive and memory subsets

The isolation of splenic CD4 T cells (\(>90\%\) pure) from BALB/c and HA-TCR mice has been described previously (8, 20). CD4 T cells were fractionated into naive (CD45RB\(^{\text{HI}}\)) and memory (CD45RB\(^{\text{LO}}\)) CD4 T cells by automated magnetic separation using the AutoMACS system (Miltenyi Biotec, Auburn, CA). Briefly, CD4 T cells were incubated with PE-conjugated CD45RB Ab (BD Pharmingen), followed by anti-PE magnetic microbeads (Miltenyi Biotec). The cells were separated by automated passage over a ferromagnetic column, followed by serial elutions resulting in CD45RB\(^{\text{HI}}\) (\(>99\%\) pure) and CD45RB\(^{\text{LO}}\) (\(>98\%\) pure) populations.

In vitro generation of effector CD4 T cells

For generation of Ag-activated effector CD4 T cells (Ag effectors), HA-TCR CD4 T cells (1 \(\times\) 10\(^6\) cells/ml) were incubated with 5 \(\mu\)g/ml HA peptide-treated and mitomycin C-treated (Roche, Indianapolis, IN) T-depleted splenic APC (20) (5 \(\times\) 10\(^5\) cells/ml) for 5 days at 37\(^\circ\)C. For generation of anti-CD3 activated effector T cells, BALB/c CD4 T cells (1 \(\times\) 10\(^6\) cells/ml) were incubated with 5 \(\mu\)g/ml soluble anti-CD3 Ab and 3 \(\times\) 10\(^6\) cells/ml APC for 4 days at 37\(^\circ\)C (20). Primary and secondary effector T cells were generated by incubating the CD45RB\(^{\text{HI}}\) or CD45RB\(^{\text{LO}}\) subset with 5 \(\mu\)g/ml soluble anti-CD3 Ab in the presence of MHC class II\(^{\text{ID}}\) or MHC class II\(^{\text{ID}}\) APC for 4 days at 37\(^\circ\)C as previously described (20) (Fig. 1). The resultant effector subsets were centrifuged through Ficoll, washed to remove dead and contaminating accessory cells, and resuspended in complete Medium as previously described (20). These effector CD4 T cells were \(>95\%\) pure.

Immunoblotting and immunoprecipitation

For immunoprecipitation and Western blot analysis, cells were incubated for 2 min at 37\(^\circ\)C in the absence (–) or the presence of anti-CD3 Ab (+) and subsequently lysed in 1\% Nonidet P-40 lysis buffer with protease/ phosphatase inhibitors as previously described (19). Protein content in cell lysates was quantitated using the detergent-compatible protein assay system (Bio-Rad, Hercules, CA). Cell lysates were resolved on reducing SDS-PAGE, and gels were electrophoretically transferred to nitrocellulose membranes. For anti-phosphotyrosine immunoblots, blocking, and Ab dilution were in PBS/T and 5\% milk. Hybridizing protein bands were detected using ECL (Amersham, Arlington Heights, IL) and were revealed with Hyperfilm ECL (Amersham). In some cases blots were stripped of bound Abs as previously described (19) before reprobing.

For immunoprecipitations and GST-Grb2 pull-down experiments, 5 \(\times\) 10\(^5\) to 10\(^6\) cells were lysed in 100 \(\upmu\)l of 1\% Nonidet P-40 lysis buffer. Lysates were precleared with protein-G Sepharose or GST-agarose for 1 h at 4\(^\circ\)C, followed by incubation with Ab preadsorbed to protein G-Sepharose, GST-agarose, or GRB2-GST-agarose for 2 h at 4\(^\circ\)C. For the anti-SLP-76 immunoprecipitation of whole CD4 T cells, negative controls with normal sheep serum (Jackson ImmunoResearch Laboratories, West Grove, PA) preadsorbed to protein G-Sepharose were also performed. Immunoprecipitates were washed and resuspended in sample buffer before analysis by SDS-PAGE and immunoblotting.

Results

Differences in SLP-76 expression in naive, memory, and effector CD4 T cells

Our previous studies of biochemical analysis of CD4 T cell subsets used the CD45RB\(^{\text{HI}}\) and CD45RB\(^{\text{LO}}\) subsets of BALB/c splenic CD4 T cells as naive and memory cells, respectively. We have extensively characterized the phenotypic and functional attributes of these subsets to confirm their naive and memory designations (8, 20), consistent with numerous studies establishing the validity of CD45 isoform expression as functionally and phenotypically delineating naive and memory T cells in mice and humans (3, 24, 25). With the goal of analyzing signaling differences in an Ag-specific system, we isolated highly purified naive (CD45RB\(^{\text{HI}}\)) and memory (CD45RB\(^{\text{LO}}\)) CD4 T cells from HA-TCR transgenic mice in which 30–50\% of peripheral CD4 T cells express the transgene-encoded TCR (clonotype 6.5) specific for influenza HA peptide and I-E\(^d\) (21). HA-specific effector CD4 T cells were generated in vitro by activation of HA-TCR CD4 T cells with HA peptide and APC (Fig. 1). We previously demonstrated that the HA-TCR CD45RB\(^{\text{HI}}\), CD45RB\(^{\text{LO}}\), and in vitro activated cells exhibit all the functional attributes of naive, memory, and effector CD4 T cell subsets, respectively, in their responses to HA peptide Ag (5, 20).

We compared the patterns of total tyrosine phosphorylation in lysates of resting and CD3 cross-linked HA-TCR naive, effector, and memory T cells by anti-phosphotyrosine immunoblotting (Fig. 2a). Naive CD4 T cells exhibit a tyrosine phosphorylation profile (20) (Fig. 2a, lanes 1 and 2) that resembles T cell lines and clones, whereas memory CD4 T cells exhibit greatly diminished phosphorylation overall, with a striking absence of phosphorylated species at 130 and 70–80 kDa in lysates derived from resting and activated CD4 T cells.
CD4 T cells exhibit a significanly reduced pattern of tyrosine phosphorylation: effector CD4 T cells exhibit extensive hyperphosphorylation relative to naive cells, whereas memory CD4 T cells exhibit a significantly reduced pattern of tyrosine phosphorylation.

The tyrosine phosphorylation analysis (Fig. 2A) revealed a highly phosphorylated 76-kDa band in lysates derived from anti-CD3-stimulated naive and effector CD4 T cells, but not memory T cells. Because this band corresponds in size to the critical linker/adapter molecule SLP-76 (26), we stripped and reprobed the immunoblot in Fig. 2A with antiserum for SLP-76 (22). As shown in Fig. 2A, third blot, SLP-76 protein expression was markedly reduced in memory CD4 T cells compared with the naive subset (compare lanes 3 and 4 to lanes 1 and 2). Effector cells showed overexpression of SLP-76 when cell equivalents were analyzed; however, this increase was less apparent when protein equivalents were analyzed (Fig. 2A, lanes 5–8). Despite these alterations in SLP-76 expression, naive, memory, and effector CD4 T cells express comparable levels of PLCγ (Fig. 2A, middle blot), Vav protein (data not shown), ZAP-70 (data not shown), and Erk1/2 (see Fig. 4A and data not shown). These results indicate that memory CD4 T cells exhibit a specific decrease in expression of SLP-76, while expression of other TCR-coupled signaling intermediates is unaffected.

To confirm that these results were not peculiar to HA-TCR CD4 T cells, we analyzed BALB/c-derived naive, memory, and effector CD4 T cells (20) (see Fig. 1) for expression of SLP-76, PLCγ, and Erk1/2. As shown in Fig. 2B, BALB/c-derived memory T cells likewise exhibited a dramatic reduction in SLP-76 expression, while expression of PLCγ and Erk1/2 MAP kinases was unaffected. In equivalent numbers of effector T cells, the expected up-regulation of all three of these signaling intermediates was observed. These results indicate that diminished SLP-76 protein expression is intrinsic to the memory T cell subset.

Because a fraction of SLP-76 can localize to detergent-insoluble glycolipid-enriched membrane microdomains after activation (27),
we asked whether the loss of SLP-76 from Nonidet P-40 lysates of memory CD4 T cells was due to partitioning to another cellular compartment. We thus analyzed Nonidet P-40-insoluble fractions (pellet) of all three subsets for the presence of membrane-associated SLP-76 protein and, as controls, PLC-γ protein and LAT, a T cell-specific linker/adapter protein known to partition to the membrane fraction (28). As shown in Fig. 2C, naive and effector CD4 T cells express significant levels of SLP-76 protein in the pellet fraction, yet memory CD4 T cells express very low to undetectable levels of SLP-76 protein in both Nonidet P-40-soluble and insoluble fractions. LAT protein, by contrast, is present at high levels in both soluble and pellet fractions from memory as well as naive and effector CD4 T cells. (The 40-kDa mouse LAT has decreased mobility compared with human LAT (36/38 kDa) due to the additional negative charge of the mouse protein (29). PLC-γ protein is also present in lysates and pellets from all three subsets, yet lower proportions of PLC-γ are consistently found in pellets of effector and memory T cells compared with the naive subset (Fig. 2C and data not shown). These results demonstrate that reduced SLP-76 expression in memory cell lysates is not due to partitioning to the membrane compartment, and LAT is expressed at normal levels and is appropriately partitioned in memory T cells.

SLP-76-mediated signaling and SLP-76 associations in naive, effector, and memory CD4 T cells

The low level expression of SLP-76 in memory T cells suggested two possible signaling consequences: either SLP-76 was not involved in memory T cell signaling, or the residual SLP-76 participated in memory CD4 T cell signaling through phosphorylation and/or coupling to additional signaling molecules. To address these possibilities, we investigated SLP-76 phosphorylation and protein associations using direct immunoprecipitation with anti-SLP-76 antiserum. We first wished to establish the pattern of phosphorylation of SLP-76 and associated proteins in anti-SLP-76 immunoprecipitates of resting and anti-CD3-stimulated primary mouse CD4 T cells (Fig. 3A). In SLP-76 immunoprecipitates of unstimulated CD4 T cells, weakly phosphorylated bands of 60 and 76 kDa are present (Fig. 3A, lane 5), whereas strongly phosphorylated proteins of 40 (corresponding in size to LAT), 60, 76 (SLP-76), 100, and 120 kDa are present in anti-SLP-76 immunoprecipitates of anti-CD3-stimulated CD4 T cells (Fig. 3A, lane 6). These SLP-76-associated phosphorylated proteins are not present in control immunoprecipitates with protein G-Sepharose alone or protein G-Sepharose and normal sheep serum (Fig. 3A, lanes 1–4), and are consistent with the SLP-76-associated phosphorylated proteins identified in human Jurkat T cells (30) and primary mouse thymocytes (31).

We next performed anti-SLP-76 immunoprecipitates of resting and anti-CD3-stimulated naive, effector, and memory CD4 T cell subsets and found differences in SLP-76 expression, phosphorylation, and association to phosphorylated proteins. Consistent with results obtained with anti-SLP-76 Western blots of whole cell lysates (Fig. 2), we found substantially reduced levels of SLP-76 protein in anti-SLP-76 immunoprecipitates derived from memory CD4 T cells, compared with naive and effector cells (Fig. 3B, second blot, compare lanes 3 and 6 to lanes 3, 4, 9, and 10). Following anti-CD3 stimulation, four main phosphorylated species are present in anti-SLP-76 immunoprecipitates of lysates derived from anti-CD3-stimulated naive and effector CD4 T cells (Fig. 3A, lanes 3, 4, and 9–12): a 76-kDa protein corresponding to SLP-76, as confirmed by the anti-SLP-76 Western blot (Fig. 3B, second blot), a 40-kDa protein corresponding to LAT (confirmed by an anti-LAT Western blot; data not shown), and phosphorylated proteins of 34 and 120 kDa. All four of these proteins are more highly phosphorylated in stimulated effector T cells, although a 60-kDa protein present in resting and stimulated naive and memory T cells is not present in effector T cell lysates (Fig. 3B, lanes 2–6 and 9–12). In anti-CD3-stimulated memory T cells, by contrast, SLP-76 is weakly phosphorylated and associated to weakly phosphorylated LAT (40 kDa) and p34, but not to phosphorylated p120 (Fig. 3B, top blot), indicating that the low level of SLP-76 protein present in memory T cells is coupled to a subset of potential SLP-76-associated signaling molecules. When taken together, these results demonstrate that the highest levels of SLP-76 phosphorylation and coupling to phospho-LAT occur in effector T cells, followed by naive T cells, and memory T cells exhibit the lowest levels of SLP-76 phosphorylation and phospho-LAT coupling and lack the SLP-76-p120 association.
We asked whether differential SLP-76 coupling in memory T cells was due to differential association with the SLP-76-LAT linker molecule, GADS (31). As shown in Fig. 3B, bottom blot, high levels of GADS protein are present in SLP-76 immunoprecipitates from all three subsets. The substantial levels of GADS protein associated with the low levels of SLP-76 protein in memory T cells (Fig. 3B, bottom blot, lanes 5 and 6), suggest remarkably efficient SLP-76-GADS coupling in memory T cells. The association of SLP-76 and GADS in resting and activated cells is consistent with findings that the SH3 portions of GADS associate with proline-rich regions of SLP-76 (32).

Although SLP-76 is known to associate with LAT via GADS, GADS does not couple to downstream activators such as the Ras pathway (33). The ubiquitous linker adapter protein, Grb2, has been shown to bind high levels of SLP-76 and LAT in vitro (34, 35), and to couple to downstream Ras-mediated MAP kinase activation (34). We asked whether phosphorylated proteins in memory T cell lysates could potentially interact with Grb2 in GST-Grb2 “pulldown” experiments. As shown in Fig. 4A, the pattern of tyrosine-phosphorylated proteins that could associate with Grb2 differed among naive, memory, and effector CD4 T cells. In particular, memory CD4 T cells displaying a striking paucity of Grb2-coupled phosphorylated proteins compared with naive and effector CD4 T cells, particularly at the 100–120 range (Fig. 4A). The Grb2-coupled phosphorylated species in the three subsets fall into four categories: 1) those that are found in all three subsets, albeit at lower levels in memory T cells (p40 corresponding to phospho-LAT, and p50–65, shown in black), 2) those that are only expressed by naive and effector CD4 T cells (p76 corresponding to SLP-76, and p120, shown in red), 3) those species most prominent in effector CD4 T cells (p34 and p140, shown in blue), and 4) one species found most highly phosphorylated in memory CD4 T cells (p28, shown in green).

We also directly assessed the ability of SLP-76 and PLC-γ to associate with Grb2 in these pulldown experiments. As shown in Fig. 4B, lower blot, SLP-76 protein was detected in anti-SLP-76 immunoblots of Grb2-GST precipitates from naive and effector, but not memory, CD4 T cells (lanes 3–6, 9, and 10). As a control, we also looked at the association of PLC-γ with grb2, as this protein is expressed equally in naive, effector, and memory CD4 T cells and has been shown to be associated with Grb2 in cell lines (36). In naive and memory T cells, PLC-γ binds Grb2 efficiently only after CD3 stimulation (Fig. 4B, upper blot, lanes 4 and 6), whereas in effector cells, high levels of PLC-γ efficiently bind Grb2 in the absence or the presence of CD3 stimulation (lanes 9 and 10). When taken together, the SLP-76 immunoprecipitation and Grb2 association analyses indicate that in memory T cells, SLP-76 can couple to GADS, but lacks accessibility to the Grb2 linker/adaptor, whereas in naive and effector T cells, SLP-76 binds GADS and is accessible to Grb2.

**Downstream activation in naive, memory, and effector CD4 T cells**

We asked whether the greatly diminished Grb2-interacting phosphorylated proteins and/or lower SLP-76 expression in memory T cells affect its ability to activate downstream MAP kinases. Reduced Erk1/2 MAP kinase activation was found in SLP-76-deficient Jurkat T cells (37), suggesting that memory T cells may display similar impairments. We therefore probed lysates derived from resting and activated naive, effector, and memory CD4 T cells for the presence of phosphorylated Erk1/Erk2 kinases (Fig. 5A). While naive, memory, and effector CD4 T cells display similar kinetics of peak Erk1/2 phosphorylation (2 min) and dephosphorylation (5–10 min), unstimulated effector T cells exhibit simultaneous Erk1/2 phosphorylation that is further increased upon stimulation. Memory T cells phosphorylate Erk1/2 following TCR/CD3 cross-linking (Fig. 5A, lanes 6–8) at levels slightly lower than stimulated naive cells (lanes 2–4), yet these levels are remarkably robust considering the minimal SLP-76 expression and diminished upstream phosphorylation events in memory T cells.

Because downstream memory T cell signaling appeared comparable to naive T cells, we assessed whether memory cells could likewise differentiate into effector cells expressing high levels of SLP-76 and hyperphosphorylated Erk kinase. We generated primary and secondary effector CD4 T cells by anti-CD3 stimulation of BALB/c naive and memory CD4 T cells, respectively, as demonstrated in Fig. 1. We have previously shown that the functional
and phosphotyrosine profiles of primary and secondary effector T cells are indistinguishable (20). In this study, we demonstrate that memory cell-derived secondary effector CD4 T cells express high levels of SLP-76 protein (Fig. 5B, lanes 7, 8) and Erk1/2 hyperphosphorylation similar to primary effector CD4 T cells (Fig. 5B, lanes 3 and 6). These results demonstrate that memory CD4 T cells with low level SLP-76 expression have low level Erk expression (third blot), and SLP-76 expression (second blot) persist in effector T cells rested up to 2 days (lanes 5–8). (Beyond 2–3 days in medium alone, substantial death of effector cells precluded further biochemical analysis). These results indicate that the signaling alterations in effector T cells are not due to acute activation and that rested effector cells maintain their hyperphosphorylation capacity. Whether effector cells will eventually revert to a memory-specific signaling pattern in vivo remains to be established.

**Discussion**

We demonstrate in this study that naive, effector, and memory CD4 T cells exhibit distinct TCR-coupled signaling configurations based on differences in the expression and activation of key TCR-coupled signaling intermediates. In Fig. 7, we present a schematic model for TCR-coupled signaling in naive, effector, and memory CD4 T cells. Naive CD4 T cells follow the generalized scheme of TCR signal transduction established from study of T cell lines and knockout models (39), with TCR ligation leading to activation of CD3ζ-associated ZAP-70 kinase (19), which, in turn, phosphorylates the T cell-specific adapter molecules SLP-76 and LAT. Phosphorylated LAT linked to SLP-76 via GADS can also bind to Grb2, resulting in Ras-mediated MAP kinase activation (40).
In contrast to naive CD4 T cells, memory CD4 T cells appear to lack certain key TCR-coupled proximal and intermediate signaling events (Fig. 7). While CD3ζ is phosphorylated following TCR/CD3 cross-linking, CD3ζ-associated ZAP-70 is not (19). Downstream of ZAP-70, memory CD4 T cells exhibit a dramatic reduction in SLP-76 expression, concomitant decreases in SLP-76 phosphorylation, and few phosphorylated proteins that can efficiently couple to Grb2 (see Fig. 6). Despite these signaling diminutions, the residual memory cell SLP-76 couples efficiently to high levels of GADS, resulting in associations to phospho-LAT that can subsequently link to Grb2 and lead to MAP kinase activation. We predict that in the absence of ZAP-70 activation, p56Lck may phosphorylate LAT and residual SLP-76 (Fig. 7).

Effector CD4 T cells exhibit considerable augmentation of signaling characterized by overall hyperphosphorylation. In particular, both GADS-associated SLP-76 and LAT are highly phosphorylated in effector cells and coupled to Grb2 with additional phosphorylated proteins (p34, p140), resulting in hyperphosphorylation of the MAP kinases Erk1/2 before and after TCR/CD3 cross-linking (Fig. 7).

The amplification of TCR-mediated signals in effector cells stands in stark contrast to the reduced level of signaling observed in memory CD4 T cells. This signaling dichotomy appears to contradict the similar functional outcomes and enhanced responses known to characterize both effector and memory T cells (3). We propose that distinct signaling mechanisms in effector and memory T cells both enable efficient responses to TCR triggering, yet differentially regulate their activation properties. In effector T cells, the increased phosphorylation and recruitment of signaling molecules accounts for their heightened functional responses and rapid activation kinetics (2, 5) and are consistent with findings demonstrating increased recruitment of the p56Lck kinase in effector CD8 T cells (41). Moreover, increased Ca²⁺ flux and IL-2 production in effector T cells (41, 42) may be due to augmentations of SLP-76-coupled signaling, because Ca²⁺ flux and IL-2 production in Jurkat cells are affected by alterations in SLP-76 expression (34, 37). These amplifications in downstream signaling and IL-2 production may ultimately drive effector T cells toward AICD, as both MAP kinase activation and treatment of activated T cells with IL-2 have been shown to lead to apoptosis (43, 44).

In memory T cells, the reductions in TCR-signaling events may have two consequences. First, fewer signaling linkages may result in efficient and direct coupling to downstream activators, leading to enhanced responses relative to naive T cells (45) via a shorter biochemical route. For example, the minimal SLP-76/LAT linkage in memory CD4 T cells appears sufficient to couple TCR triggering to downstream MAP kinase activation even in the absence of additional SLP-76 associations. Second, based on results showing that SLP-76-deficient Jurkat T cells exhibit diminished Ca²⁺ flux and IL-2 production (37), it is likely that reduced SLP-76 expression in memory CD4 T cells accounts for the lower Ca²⁺ flux and IL-2 production previously identified in memory vs naive CD4 T cells (46, 47). These reductions in overall signaling and IL-2 production may protect activated memory cells from AICD (48), therefore promoting a longer life span in vivo.

It remains to be determined whether the observed differences in SLP-76 expression and MAP kinase activation in naive, effector, and memory T cells are directly linked to specific functions such as cytokine production, and whether the expression and activation state of these critical intermediates are responsible for the generation and/or maintenance of memory T cells. These important issues will need to be addressed in vitro using retroviral-mediated gene transduction into primary subsets of T cells, and in vivo through the design of new mouse models involving the selective expression of these molecules before and after T cell activation.

The reduction of SLP-76 expression in memory CD4 T cells appears to occur post-transcriptionally, as we have detected comparable amounts of SLP-76 transcripts by RT-PCR in naive, effector, and memory subsets (data not shown). SLP-76 may be selectively degraded in memory T cells, as its N-terminus contains a single amino-terminal proline-glutamic acid-serine threonine-rich domain that plays a role in maintaining the metabolic stability of proteins (22). We are currently examining whether the level of SLP-76 expression and MAP kinase activation in naive, effector, and memory T cells are directly linked to specific functions such as cytokine production, and whether the expression and activation state of these critical intermediates are responsible for the generation and/or maintenance of memory T cells. These important issues will need to be addressed in vitro using retroviral-mediated gene transduction into primary subsets of T cells, and in vivo through the design of new mouse models involving the selective expression of these molecules before and after T cell activation.

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How do these memory CD4 T cell-specific alterations in signaling arise? One explanation for this phenomenon is that prolonged homeostatic turnover in vivo driven either by cytokine stimulation (49) and/or interactions with cross-reactive Ags may tonically down-regulate signaling in memory T cells. A second

FIGURE 7. Schematic model for TCR-coupled signaling in naive, effector, and memory CD4 T cells. For explanation, see text.
Differential Signaling in Effector and Memory T Cells

possibility is that the CD45 isoform expressed by memory T cells (CD45RO) plays a role in TCR-mediated signaling due to its close association with the TCR/CD3 complex (50) and its intracellular phosphorylation portion. For example, reduced SLP-76 phosphorylation and coupling to Vav were observed in Jurkat cells expressing the CD45RO isoform, but not in Jurkat cells expressing higher m.w. CD45 isoforms (51). However, we think it less likely that these signaling differences are solely due to CD45 isoform expression, as CD45RO-expressing effector T cells also exhibit hyper-phosphorylation (52).

Our findings that effector T cells maintain hyperphosphorylation of Erk1/2 kinases during prolonged removal from the activating stimulus suggest that a heightened signaling state is a stable feature of effector T cell differentiation and does not require continuous stimulation. We thus propose that phospho-Erk and SLP-76 can serve as useful biochemical markers for effector and memory CD4 T cells that are not readily distinguished by current phenotypic and functional criteria. We have used biochemical analysis of total tyrosine phosphorylation to detect an effector-like subset within the long-lived memory T cell pool (20), and effector-memory subsets have also been detected in humans based on activation kinetics (24). The biochemical differences in intracellular signaling presented in this study can provide insight into the function of effector and memory subsets and serve as novel criteria to analyze the composition of the memory T cell pool.

Acknowledgments
We thank Drs. Gary Koretzky and Ronald Wange for helpful discussions with regard to this work, and Drs. Jan Cerny, Kristin Abraham, Sandeep Krishnan, and Gregg Hadley (all from University of Maryland, Baltimore, MD) for critical reading of this manuscript.

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Received 16:1303. 2001. Vi-


