A Biochemical Signature for Rapid Recall of Memory CD4 T Cells[1]

Meena R. Chandok, Francesca I. Okoye, Modesta P. Ndejembi, and Donna L. Farber[2]

Mechanisms for the rapid recall response mediated by memory T cells remain unknown. In this study, we present a novel, multiparameter analysis of TCR-coupled signaling and function in resting and activated naive and memory CD4 T cells, revealing a biochemical basis for immunological recall. We identify a striking elevation in expression of the proximal tyrosine kinase Zap70 in resting Ag-specific and polyclonal mouse memory vs naive CD4 T cells that is stably maintained independent of protein synthesis. Elevated Zap70 protein levels control effector function as IFN-γ production occurs exclusively from the Zap70^{high} fraction of activated T cells in vitro and in vivo, and specific down-modulation of Zap70 expression in memory CD4 T cells by small interfering RNA or protein inhibition significantly reduces rapid IFN-γ production. Downstream of Zap70, we show quantitative differences in distal phosphorylation associated with effector function in naive and memory subsets, with low accumulation of phosphorylation in memory T cells producing IFN-γ at early time points, contrasting extensive phosphorylation associated with IFN-γ production following sustained activation of naive T cells. Our results reveal a novel biochemical signature imparted to memory CD4 T cells enabling efficacious responses through increased Zap70 expression and reduced accumulation of downstream signaling events. ***The Journal of Immunology,*** **2007, 179: 3689–3698.**

Immune memory is characterized by efficacious responses to previously encountered Ags, mediated by memory T lymphocytes that produce effector cytokines immediately upon antigenic challenge. Although the enhanced activation properties and potent functional capacities of memory CD4 and CD8 T cells are well-documented (1–3), the mechanisms by which TCR engagement is coupled intracellularly to rapid effector responses remain unknown. TCR-coupled intracellular signaling events have been extensively characterized in T cell lines and unfractionated primary T cells, revealing a progression of intracellular events linking TCR ligation to nuclear gene transcription (4). Initial phosphorylation of the TCR-associated CD3ε and CD3ζ subunits by the proximal p56^{lck} kinase results in the recruitment, phosphorylation, and activation of the 70-kDa Src homology 2(SH2)-containing Zap70 tyrosine kinase (5, 6). Zap70 subsequently phosphorylates the linker-adapter molecules SH2-containing leukocyte molecule of 76kDa (SLP-76) and linker for activation of T cells (LAT) which serve as scaffolds for assembly of a signaling cluster triggering downstream MAPK activation, calcium flux, and *IL-2* gene transcription (7). The participation of these TCR-coupled signaling intermediates in the distinct responses of naive vs memory T cells and in coupling to effector cytokine production remains undefined (8).

In this study, we hypothesized that the capacity of memory T cells to elicit rapid recall responses is mediated by alteration(s) in TCR-coupled signaling that are stably maintained in the resting state, and likely associated with acquisition of effector function during activation and differentiation of naive T cells. We had previously applied standard biochemical approaches to analyze TCR-coupled signaling in lysates derived from polyclonal naive and memory CD4 T cells isolated by cell surface phenotype, and had found decreased intracellular phosphorylation and coupling to linker adapter molecules in the memory subset (9–11), although how these signaling changes coupled to rapid recall remained unknown. Thus, to investigate the signaling mechanisms for rapid recall, we took advantage of new reagents and multiparameter approaches for high resolution analysis of signal transduction molecules in the native state at the single-cell level (12, 13). By applying a novel combination of single-cell signaling analysis with intracellular cytokine staining, we examined signaling events in conjunction with cytokine production in Ag-specific and polyclonal naive and memory CD4 T cells in the resting state and following antigenic stimulation at different kinetic time points in vitro and in vivo.

In this study, we report a striking and specific elevation in expression of the Zap70 protein tyrosine kinase in resting Ag-specific and polyclonal mouse memory compared with naive CD4 T cells, and distinct signaling pathways coupled to effector function in these subsets. High-level Zap70 expression in memory CD4 T cells is stably maintained independent of protein synthesis, whereas low Zap70 expression in naive T cells increases with sustained (24–48 h) antigenic stimulation requiring new protein synthesis. We establish that Zap70 protein levels control effector function, as acquisition of effector function occurs only from activated T cells that have up-regulated Zap70 expression to high levels, and conversely, specific down-modulation of Zap70 expression in memory CD4 T cells by (siRNA)[3]-mediated knockdown or specific inhibitors reduces rapid effector function. Downstream of Zap70, we show quantitative differences in the accumulation of

Division of Transplantation, Department of Surgery, School of Medicine, University of Maryland, Baltimore, MD 21201

Received for publication February 27, 2007. Accepted for publication July 1, 2007.

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 AI42092 awarded to D.L.F.

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

[3] Abbreviations used in this paper: siRNA, small interfering RNA; ICS, intracellular cytokine staining; PLC, phospholipase C; CHX, cycloheximide.

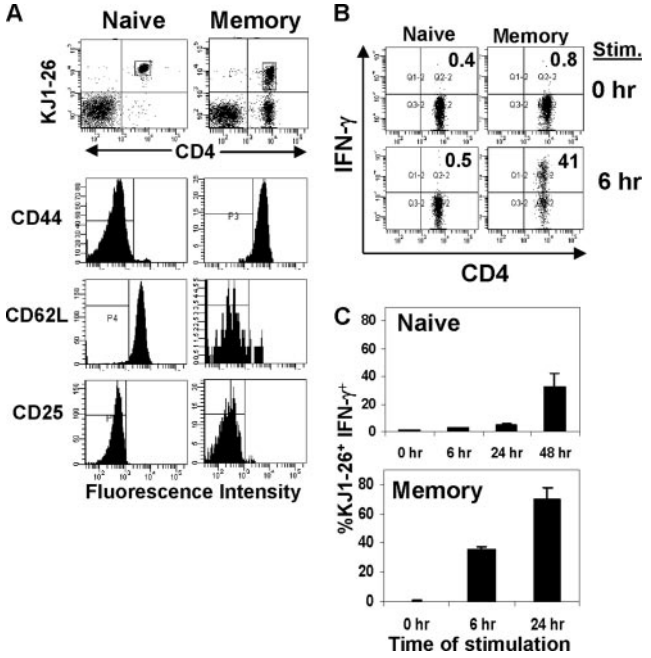

FIGURE 1. Phenotypic and functional comparison of OVA-specific naive and memory CD4 T cells. *A*, Cell surface expression of CD44, CD62L, and CD25 on resting OVA-specific naive CD4 T cells isolated from DO11.10 × RAG2$^{-/-}$ mice and memory CD4 T cells isolated from RAG2$^{-/-}$ adoptive hosts >2 mo posttransfer of primed DO11.10 effector CD4 T cells. Histograms are gated on KJ1-26$^+$CD4$^+$ T cells. Data are representative of >10 independent experiments. *B*, IFN-γ production from resting and Ag-stimulated naive and memory CD4 T cells. OVA-specific naive and memory CD4 T cells were activated with OVA peptide and splenic APC for 6 h in the presence of monensin, and cytokine production was assessed by intracellular staining. Plots shown are gated on KJ1-26$^+$ cells, with quadrants drawn based on isotype controls. *C*, Kinetics of IFN-γ production by Ag-stimulated naive and memory CD4 T cells. OVA-specific naive and memory CD4 T cells were activated as in *B* for 6–48 h and IFN-γ was assessed by ICS. The range in IFN-γ production from Ag-stimulated memory CD4 T cells was 20–40% at 6 h and 40–80% at 24 h in 15 different experiments.

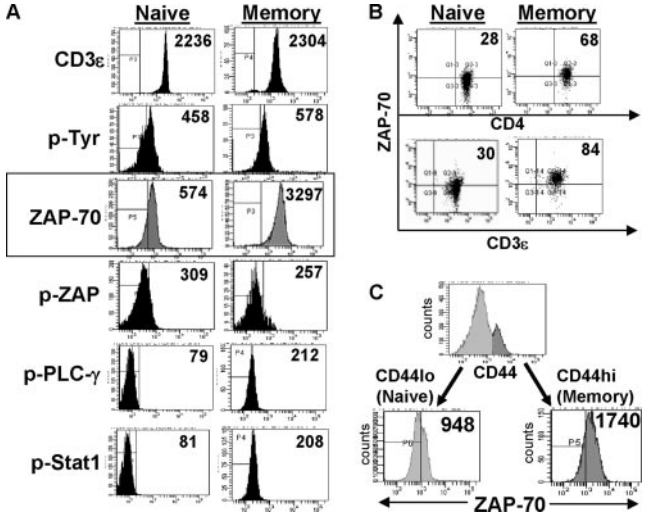

FIGURE 2. Expression of intracellular signaling molecules in resting naive and memory CD4 T cells. *A*, OVA-specific naive and memory CD4 T cells isolated as in Fig. 1 were stained intracellularly for CD3ε, total tyrosine phosphorylation (p-Tyr), Zap70 protein, phosphorylated Zap70 (p-ZAP), phosphorylated PLC-γ (p-PLC-γ), and phosphorylated Stat1 (p-Stat1). Histograms shown are gated on CD4$^+$KJ1-26$^+$ cells, with markers drawn based on isotype controls, and mean fluorescence intensity (MFI) values are shown as *inset*. *B*, Coexpression of Zap70 and CD4 (*upper panels*) or Zap70 and CD3ε (*lower panels*) in resting naive and memory CD4 T cells gated on CD4$^+$KJ1-26$^+$ cells, with markers drawn based on isotype controls. Numbers in quadrants represent the percentage of Zap70^{high}CD4$^+$ or Zap70^{high}CD3ε$^+$. *C*, Expression of Zap70 in polyclonal naive (CD44^{low}) and memory (CD44^{high}) CD4 T cells. Results show CD44 expression of polyclonal CD4 T cells from BALB/c mice, with Zap70 histograms gated on CD4$^+$CD44^{low} (naive) and CD4$^+$CD44^{high} (memory) populations. Numbers in histograms indicate MFI. Data are representative of three independent experiments.

distal phosphorylation events associated with effector function in naive and memory subsets in vitro and in vivo. Our findings reveal a biochemical basis for rapid recall by memory T cells, and also identify a new mechanism for control of TCR-coupled signaling and function via alterations in proximal kinase expression at the protein level.

Materials and Methods

Mice

BALB/c mice (8–16 wk of age) were obtained from the National Cancer Institute Biological Testing Branch. DO11.10 × RAG2$^{-/-}$ mice (Taconic Farms), DO11.10 mice (14) bred as heterozygotes onto BALB/c backgrounds, and RAG2$^{-/-}$ mice (15) on BALB/c backgrounds (Taconic Farms) were maintained in the Animal Facility at the University of Maryland Medical Center (Baltimore, MD) under specific pathogen-free conditions.

Reagents and Abs

The following Abs were purified from bulk culture supernatants and purchased from BioExpress: anti-CD8 (TIB 105), anti-CD4 (GK1.5), anti-I-A^d (212.A1), and anti-Thy-1 (TIB 238). Fluorochrome-conjugated Abs directed against CD4 (clone GK1.5), CD25 (PC61), CD69 (H1.2F3), CD62L (MEL-14), CD44 (IM7), CD3ε (145-2C11), IFN-γ (XMG1.2), phospho-Zap70 (17-P), phospho-phospholipase C (PLC)-γ1 (27/PLC), and phospho-Stat1 (clone 4a) were all purchased from BD Pharmingen. PE- and allophycocyanin-conjugated KJ1-26 specific for the DO11.10 TCR clonotype, PE-, and FITC-conjugated Zap70 (1E7.2) were obtained from Invitrogen Life Technologies; FITC-conjugated phosphotyrosine (PT-66) was obtained from Sigma-Aldrich; and phospho-p38 (28B10) and phospho-erk1/2 (E10) were purchased from Cell Signaling Technology. OVA peptide (323–339, ISQAVHAAHAEINEAGR) was synthesized by the Biopolymer Laboratory at the University of Maryland School Of Medicine.

Isolation of naive and memory CD4 T cells

OVA-specific naive CD4 T cells were isolated from spleens of DO11.10 × RAG2$^{-/-}$ mice by immunomagnetic depletion (16), or by direct sorting of Thy1$^+$ T cells using anti-CD90-conjugated magnetic beads (Miltenyi Biotec) and the autoMACS for analysis by real-time PCR, Western blotting, and by confocal microscopy. Generation of OVA-specific memory CD4 T cells in vivo was accomplished using an adoptive transfer system extensively validated in the laboratory (16–20). Briefly, in vitro-primed DO11.10 effector cells activated for 2 days (unless specified) were transferred into RAG2$^{-/-}$ adoptive hosts (5 × 10^6/mouse) and persisting memory CD4 T cells were harvested from spleen after 2–5 mo posttransfer for subsequent analysis (see below).

Western blotting

For Western blot analyses, T cells (2 × 10^6) were lysed in SDS sample buffer with protease/phosphatase inhibitors and were probed with primary (CD3ζ, Zap70) and HRP-conjugated secondary Abs as described (10, 11). Blots were scanned using a Canon flatbed scanner (LiDE60) and densitometric analysis of the autoradiograms was performed with the 1Dscan Ex3.1 Evaluation system (Scanalytics).

Quantitative real-time PCR

Primers for detection of CD4, Zap70, GAPDH, and hypoxanthine phosphoribosyltransferase mRNAs were designed using the Primer Express 2.0 program (Applied Biosystems). Total cDNA was isolated from CD90-sorted naive and memory CD4 T cells using the cDNA synthesis kit from NEB. Total cDNA (30 ng) was used as starting material for real-time PCR

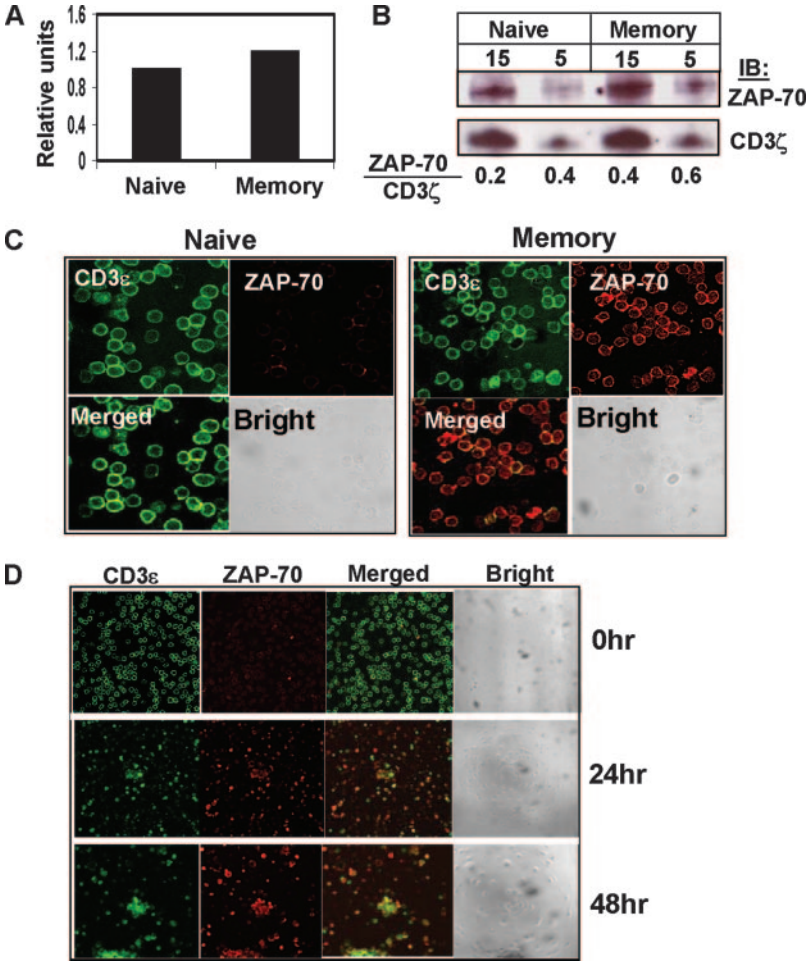

FIGURE 3. Regulation of Zap70 expression in resting CD4 T cells. *A*, Real-time PCR analysis of Zap70 transcript expression in OVA-specific naive and memory CD4 T cells purified to >95% by Thy1$^+$ sorting expressed as a ratio of Zap70:CD4 transcripts. *B*, Western blot analysis of Zap70 and CD3ζ protein expression in OVA-specific naive and memory CD4 T cells, in different dilutions of cell lysates. The densitometric ratio of Zap70:CD3ζ expression in naive and memory subsets is indicated at the *bottom*. *C*, Confocal analysis of CD3ε and Zap70 expression in highly purified Thy1-sorted OVA-specific naive and memory CD4 T cells. Images show CD3ε (green), intracellular Zap70 (red), and merged images with yellow areas denoting coassociation of the two molecules. Results are representative of two independent experiments. *D*, Confocal analysis of Zap70 and CD3ε expression in resting and activated naive CD4 T cells. Expression of CD3ε, and Zap70 on purified OVA-specific naive CD4 T cells before and after stimulation with OVA/APC for 24 and 48 h, were analyzed by confocal microscopy. Images show CD3ε (green), intracellular Zap70 (red), and merged images with yellow areas denoting coassociation of the two molecules. Results are representative of two independent experiments.

quantitation with SYBR Green (Applied Biosystems) on an Applied Biosystems 7900HT. Cycle threshold values were compared using the Δ-Δ_{CT}-cycle threshold method using hypoxanthine phosphoribosyltransferase and *GAPDH* as a housekeeping gene (21).

Intracellular cytokine staining (ICS)

ICS analysis of signaling intermediates and cytokines was performed as previously described (17, 19). Briefly, naive or memory CD4 T cells were cultured with APC and 800 ng/ml OVA peptide for time points of 0–48 h, and monensin (Golgistop; BD Pharmingen) was added 6 h before harvesting. Cells were stained with Abs for surface markers CD4, CD25, CD44, KJ1-26, fixed (Cytofix buffer; BD Pharmingen), permeabilized, and stained intracellularly with fluorochrome-conjugated Abs to cytokines, signaling intermediates, and analyzed by flow cytometry.

In vivo stimulation of naive and memory CD4 T cells

Mice were administered 6 μg of anti-CD3 Ab (C363.29B) or murine IgG2a as described (22). Spleens were removed after 4 h and splenocytes incubated in complete Clicks medium in the presence of monensin (Golgistop; BD Pharmingen) for an additional 2 h, followed by surface and intracellular staining, and were analyzed by flow cytometry.

Flow cytometry

Six-color flow cytometry was performed on a LSR II flow cytometer (BD Biosciences) using FITC, PE, PerCP, and PE-Cy7, allophycocyanin-Cy7, and allophycocyanin as fluorochromes. CD4 T cells stained with single-color fluorochromes were used for compensation, background values were established with isotype controls, and data were analyzed using FACSDiva software (BD Biosciences). For most analyses, a given marker was analyzed using several different fluorochromes to eliminate errors based on compensation.

Confocal microscopy

Naive and memory CD4 T cells were purified by sorting with anti-CD90 beads (Miltenyi Biotec) and were adhered to slides. Cells were surface stained for CD3ε, then permeabilized and stained for Zap70 or isotype controls. Cells were analyzed with a confocal microscope (Zeiss LSM510 META confocal microscope).

Inhibitor assays

Naive or memory CD4 T cells (1×10^6) were cultured with APC (3×10^6) in total volume of 1 ml of Clicks medium containing 800 ng/ml OVA peptide for time points of 0–24 h along with inhibitors cycloheximide (50 μg/ml; Sigma-Aldrich) or piceatannol (12 μM 3,4,3′,5′-tetrahydroxy-trans-stilbene; Calbiochem). Solvent alone served as a vehicle control.

siRNA-mediated knockdown

Cy3-labeled Zap70-specific siRNA or control GAPDH siRNA obtained from Ambion was delivered at a final concentration of 5 μM into 1.0×10^6 purified resting memory OVA-specific CD4 T cells using the mouse T cell nucleofector kit (Amaxa) according to the manufacturer's instructions and similar to the approach previously used for siRNA transfections in mouse T cells (23). Following nucleofection, cell suspensions were cultured in medium in 24-well plates with APC (2×10^6/well) and OVA peptide (800 ng/ml), followed by incubation at 37°C for 18 h. After 18 h, Golgistop (0.66 μl/ml culture) was added, cells were incubated for an additional 6 h, and subsequently stained for surface expression of CD4, KJ1-26 and CD25, and intracellular expression of Zap70 and IFN-γ as above.

Results

Phenotype and function of Ag-specific naive and memory CD4 T cells

For investigating the functional coupling of signaling pathways in Ag-specific naive and memory CD4 T cells, we used DO11.10 TCR-transgenic CD4 T cells expressing the KJ1-26 TCR clonotype specific for an OVA peptide (OVA). Naive, OVA-specific CD4 T cells were obtained from DO11.10 $\times$ RAG2$^{-/-}$ mice,

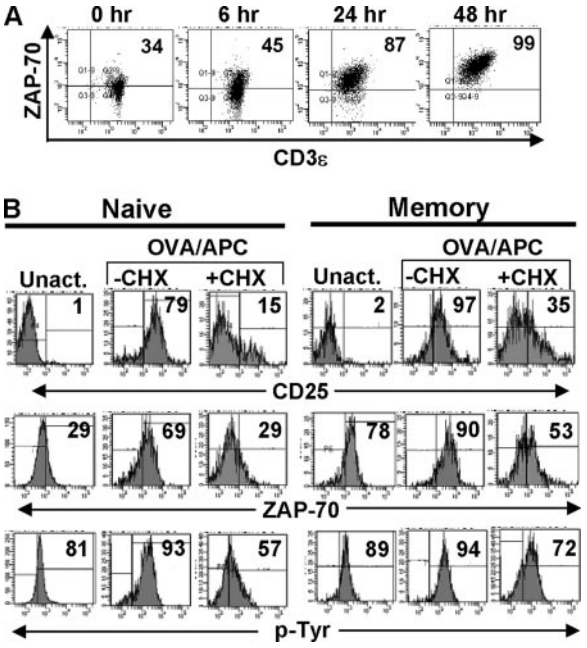

FIGURE 4. Regulation of Zap70 expression in Ag-stimulated naive and memory CD4 T cells. *A*, Changes in expression of $Zap70^{+}CD3\varepsilon^{+}$ in OVA-specific naive CD4 T cells in the resting state and following stimulation with OVA/APC for 6–48 h. Plots are gated on live KJ1-26^{+} cells, with quadrants drawn based on isotype controls and the number in the *rightmost quadrant* indicates percentage of $Zap70^{+}CD3\varepsilon^{+}$ cells. Results are representative of three independent experiments. *B*, Elevated Zap70 expression during activation requires protein synthesis in naive, but not in memory, CD4 T cells. Histograms show surface CD25 expression and intracellular Zap70 expression and total tyrosine phosphorylation (p-Tyr) in naive and memory CD4 T cells activated for 24 h with OVA/APC in the presence of CHX or vehicle control. Numbers indicate percentage positive, based on isotype controls (indicated by markers) for each parameter. Viability after 24 h in CHX averaged 70%. Results are representative of three independent experiments.

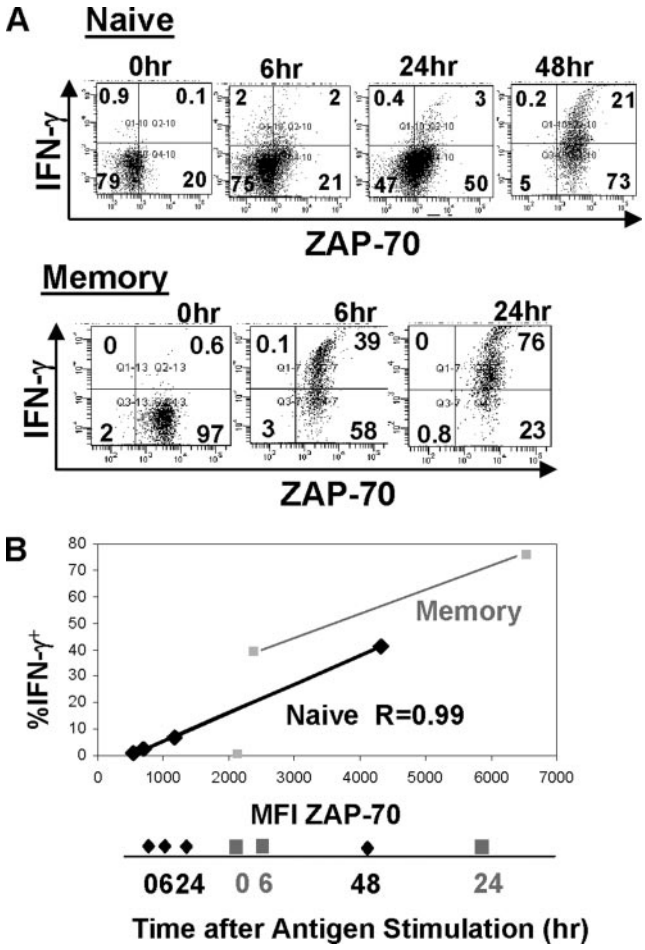

FIGURE 5. Elevated Zap70 expression correlates with the acquisition of effector function. *A*, Intracellular Zap70 vs IFN-γ production in resting (0 h) and after different periods of stimulation with OVA/APC of naive (6, 24, 48 h) and memory (6, 24 h) CD4 T cells. Results are gated on $CD4^{+}$KJ1-26^{+} cells, and number in each quadrant indicates the percentage of the respective population. Results are representative of three independent experiments. *B*, Correlation of $Zap70^{high}$ expression with IFN-γ production, collated from three independent experiments.

exclusively expressing KJ1-26^{+} CD4 T cells bearing a $CD25^{low}$/$CD44^{low}$/$CD62L^{high}$ naive phenotype (Fig. 1*A*). OVA-specific memory CD4 T cells were generated by in vitro priming of CD4 T cells obtained from DO11.10 mice (on a BALB/c background) with OVA peptide and splenic APC (OVA/APC) followed by in vivo adoptive transfer into $RAG2^{-/-}$ hosts based on a system extensively validated in the laboratory (17–20, 24, 25). The resultant memory T cells exhibit the phenotype, function, and activation properties of memory CD4 T cells similarly generated in intact BALB/c hosts or by in vivo priming (17–19, 25). The KJ1-26^{+} OVA-specific memory CD4 T cells generated here are CD25low, CD44high, and predominantly $CD62L^{low}$ (Fig. 1*A*) (KJ1-26^{-} CD4 T cells in memory hosts derive from carryover and expansion of transferred T cells with endogenous receptors). Functionally, they exhibit rapid production of IFN-γ following 6 h of antigenic stimulation (Fig. 1*B*, *right*), whereas naive CD4 T cells do not produce IFN-γ at this early time point (Fig. 1*B*, *left*). Kinetic analysis of IFN-γ production from OVA-specific naive and memory CD4 T cells shows that naive DO11.10 CD4 T cells require 48 h of sustained Ag activation to produce IFN-γ similar to memory CD4 T cells activated with Ag for only 6 h, with nearly all memory CD4 T cells exhibiting effector function (80%) at 24 h post-Ag recall (Fig. 1*C*). After 48 h of Ag stimulation, there is substantial attrition of memory T cells (16) and this time point is not included in this study.

Expression of signaling intermediates in resting naive and memory CD4 T cells

We performed an extensive analysis of the expression and phosphorylation state of signaling intermediates in resting OVA-specific naive and memory CD4 T cells using multiparameter flow cytometry (see *Materials and Methods*) to test our hypothesis that qualitative and/or quantitative alterations in TCR-mediated signaling in memory vs naive CD4 T cells may account for their distinct effector capacities. Representative results show expression of each signaling intermediate as histograms gated on the $CD4^{+}$KJ1-26^{+} population (see Fig. 1*A*) and quantified based on mean fluorescence intensity (Fig. 2*A*). In general, most of the signaling parameters examined did not differ significantly in resting naive and memory CD4 T cells, including expression of the TCR-coupled signaling subunit CD3ε (Fig. 2*A*, *row 1*), total intracellular tyrosine phosphorylation (p-Tyr; *row 2*), and lack of basal phosphorylation on tyrosine 319 (26) of the proximal Zap70 kinase (p-Zap70; *row 4*), p-PLC-γ (*row 5*), phosphorylated distal MAPK Erk1/2 (p42) and p38 (data not shown), and phosphorylated transcription factor stat1 (p-stat1, *row 6*). In notable contrast, expression of total Zap70 protein was significantly elevated in memory compared with naive CD4 T cells, manifested by a 5-fold increase

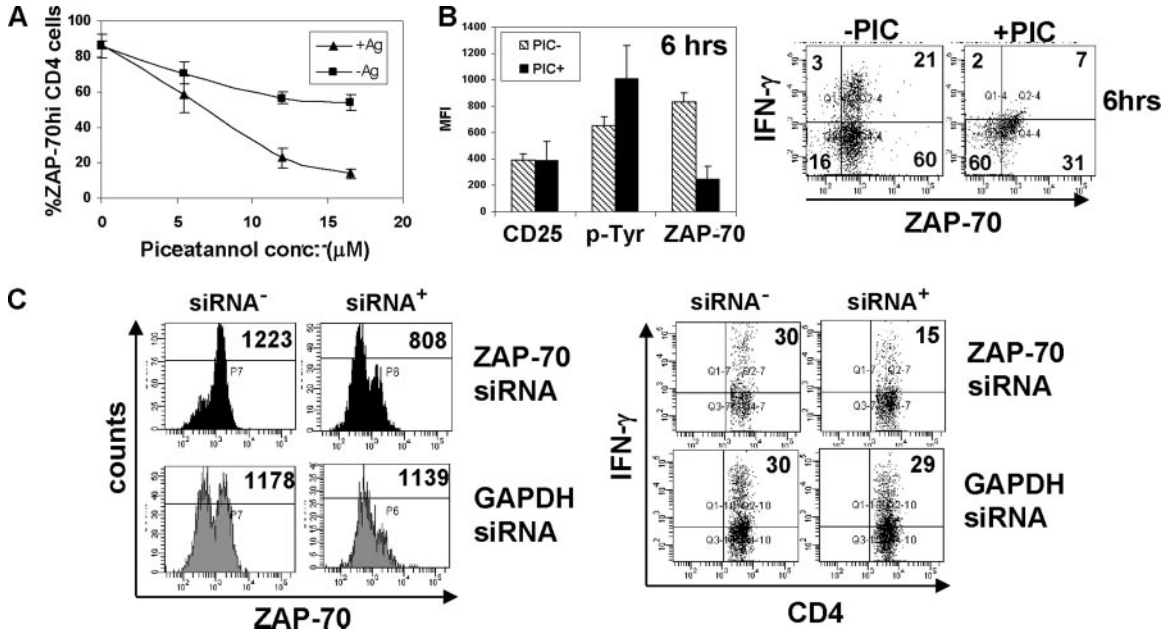

FIGURE 6. Down-modulation of Zap70 expression in memory CD4 T cells inhibits recall IFN-γ production. *A*, Dose-response curve of piceatannol and its effect on the level of Zap70 expression in memory CD4 T cells cultured ± OVA/APC for 6 h. Memory CD4 T cells used here were isolated from RAG2$^{-/-}$ adoptive hosts >2 mo posttransfer of DO11.10 CD4 T cells primed for 2 days. *B*, *Left*, Histograms show expression of CD25, p-Tyr, and Zap70 in OVA-specific memory CD4 T cells activated with OVA/APC for 6 h, all gated on CD4$^+$KJ1-26$^+$, and expressed as MFI of each marker. *Right*, Zap70$^+$/IFN-γ$^+$ production in control (−PIC) and piceatannol-treated memory (+PIC) CD4 T cells following 6 h of activation. Quadrants were drawn based on isotype control and the number in each quadrant represents the percentage of the respective population. Data shown in *A* and *B* are from three independent experiments. *C*, siRNA-mediated knockdown of Zap70. OVA-specific memory CD4 T cells were transfected with Zap70-specific Cy3-labeled siRNA (5 μM) or Cy3-labeled control GAPDH siRNA (see *Materials and Methods*). Transfected CD4 T cells were activated with APC and OVA peptide for 24 h and intracellular Zap70 staining and IFN-γ production was measured by ICS. *Left*, Total intracellular Zap70 expression of silenced (siRNA$^+$; gated on Cy3$^+$ cells) and unsilenced (siRNA$^-$; gated on Cy3-negative cells) in Zap70 and control GAPDH siRNA-transfected cells, with MFI indicated in each plot. *Right*, IFN-γ production from silenced (siRNA$^+$) and unsilenced (siRNA$^-$) populations in Zap70- and GAPDH- siRNA transfected cells. Results are representative of two independent experiments.

in mean fluorescent intensity (Fig. 2*A*, *row 3*), with the augmented expression ranging from 3- to 5-fold in multiple experiments ($n >$ 10, data not shown). Increased Zap70 expression in memory vs naive CD4 T cells occurs in the context of comparable surface expression of CD4 and CD3ε (Fig. 2*B*), with 84% of memory CD4 T cells exhibiting a CD3ε$^+$/Zap70^{high} phenotype, compared with only 30% of naive CD4 T cells (Fig. 2*B*, *row 2*).

Similar to our findings with OVA-specific subsets, we also found higher expression of intracellular Zap70 protein in polyclonal BALB/c mouse memory (CD44^{high}) compared with naive (CD44^{low}) CD4 T cells and this quantitative increase in Zap70 expression ranged from 1.5- to 2.6-fold in polyclonal populations analyzed from multiple unmanipulated mice (Fig. 2*C* and data not shown). These results establish elevated Zap70 expression as a novel feature of Ag-specific and polyclonal mouse memory CD4 T cells, and validates the OVA-specific system for examining mechanisms for functional coupling and regulation of high Zap70 expression in memory CD4 T cells.

In contrast to the difference in Zap70 protein expression, we found comparable transcript expression of Zap70 in naive and memory CD4 T cells by real-time PCR analysis (Fig. 3*A*), indicating that increased Zap70 protein expression in memory CD4 T cells is controlled posttranscriptionally. We also used Western blot analysis to analyze Zap70 protein levels in titrated quantities of naive and memory CD4 T cell lysates, and found a relative increase in total denatured Zap70 protein levels in memory CD4 T cells (Fig. 3*B*), albeit of lower magnitude than that measured by intracellular staining of the native marker by flow cytometry, confirming the increased sensitivity of this single-cell approach for assessing signaling distinctions in primary cells as suggested by Nolan and colleagues (27).

We used confocal microscopy to examine the expression, cellular localization, and TCR/CD3ε association of native Zap70 in situ in resting naive and memory CD4 T cells (Fig. 3*C*), which likewise reveals elevated expression of the Zap70 kinase in memory compared with naive CD4 T cells, with comparable CD3ε levels. Zap70 expression in memory CD4 T cells is concentrated around the plasma membrane, with areas of CD3ε and Zap70 colocalization apparent in resting memory CD4 T cells (Fig. 3*C*, *right*) and not present in resting naive T cells (Fig. 3*C*, *left*). These results demonstrate a marked increase in Zap70 expression in situ and constitutive association of Zap70 to CD3ε in resting memory vs naive CD4 T cells.

Regulation of Zap70 protein expression following Ag activation of naive and memory CD4 T cells

Our results showing high Zap70 expression in previously primed memory T cells suggested that antigenic priming of naive CD4 T cells might also lead to increased Zap70 expression. We thus stimulated OVA-specific naive CD4 T cells with OVA/APC for time points of 6–48 h, and assessed Zap70 expression in conjunction with activation phenotype and functional parameters. Stimulation of naive T cells with OVA/APC for 6 h led to slight increases in expression of CD69, an early activation marker (39–51%, data not shown) and no significant increases in CD25 or Zap70 expression (Fig. 4*A* and data not shown). By contrast, after 24–48 h of stimulation with OVA/APC there was extensive up-regulation of CD25 and CD69 as previously described (18), and substantial increases

in the level of Zap70 protein expression with all cells exhibiting a CD3ε^+/Zap70^{high} phenotype (similar to memory T cells) after 48 h of activation (Fig. 4*A*). Confocal analysis likewise demonstrates in situ increases in Zap70 expression on Ag-stimulated naive CD4 T cells after 24–48 h (Fig. 3*D*, *column 2*) accompanied by increased colocalization of CD3ε and Zap70 similar to resting memory CD4 T cells, and enhanced clustering of Zap70^{high}/CD3ε^+ cells (Fig. 3*D*, *column 3*).

Because naive CD4 T cells require sustained activation to attain levels of Zap70 comparable to resting memory CD4 T cells, we hypothesized that Zap70 protein synthesis may be differentially regulated in these two subsets. We used the protein synthesis inhibitor cycloheximide (CHX) to examine how Zap70 expression, activation, and signaling are regulated in Ag-stimulated naive and memory CD4 T cells. Treatment of naive and memory CD4 T cells ± Ag for 6 h with CHX did not affect the resting state level of Zap70 or intracellular tyrosine phosphorylation (data not shown) while 24 h treatment showed differential effects on the two subsets. For naive T cells, antigenic stimulation for 24 h led to significant up-regulation of CD25 and Zap70 expression that was completely inhibited in the presence of CHX (Fig. 4*B*, compare *columns 2* and *3*), indicating that de novo protein synthesis is required for Zap70 up-regulation. Ag stimulation of memory CD4 T cells also resulted in CD25 up-regulation, and slight increases in the already high level of Zap70 expression (Fig. 4*B*, *column 5*). Although CHX treatment inhibited CD25 up-regulation on Ag-stimulated memory CD4 T cells, the high level of Zap70 expression was maintained on >50% of memory CD4 T cells (Fig. 4*B*, *last column*). In addition, CHX treatment also significantly inhibited increases in total intracellular p-Tyr levels observed in activated naive CD4 T cells, but did not affect p-Tyr levels in memory T cells (Fig. 4*B*, *last row*). These results indicate that maintenance of elevated Zap70 expression and basal tyrosine phosphorylation in memory CD4 T cells is only partially dependent on protein synthesis.

Coupling of elevated Zap70 expression to effector function

We asked whether elevated Zap70 expression coupled to effector function by simultaneous analysis of Zap70 expression and IFN-γ production in Ag-stimulated naive and memory CD4 T cells. Activation of naive CD4 T cells with OVA/APC for 6–48 h resulted in acquisition of IFN-γ production exclusively from the Zap70^{high} population (Fig. 5*A*), with increased IFN-γ production from Zap70^{high} compared with Zap70^{low} cells most apparent after 24 h of Ag stimulation. Analysis of the level of Zap70 vs IFN-γ production from resting and Ag-stimulated naive CD4 T cells reveals a strong correlation between increased Zap70 expression and increased effector function ($R = 0.99$, Fig. 5*B*). For memory CD4 T cells, the constitutive Zap70^{high} population produces IFN-γ rapidly, and Zap70 expression is further increased after 24 h with almost all memory CD4 T cells producing IFN-γ (Fig. 5).

To determine a mechanistic link between Zap70 expression and rapid effector capacity, we took two approaches to reduce Zap70 protein expression in memory T cells and examine functional outcome. For the first approach, we used the Syk/Zap70 tyrosine kinase inhibitor piceatannol (28) that we found reduced Zap70 expression in a dose-dependent fashion in Ag-stimulated memory CD4 T cells (Fig. 6*A*). When memory CD4 T cells were stimulated with OVA/APC in the presence of piceatannol for 6 h, Zap70 expression was specifically reduced without affecting the basal level of CD25 and total p-Tyr content (Fig. 6*B*, *left*), and resulted in a striking inhibition of rapid IFN-γ production (Fig. 6*B*, *right*). As a second approach, we used siRNA (29) to drive down expression of Zap70 in memory CD4 T cells ex vivo and determine

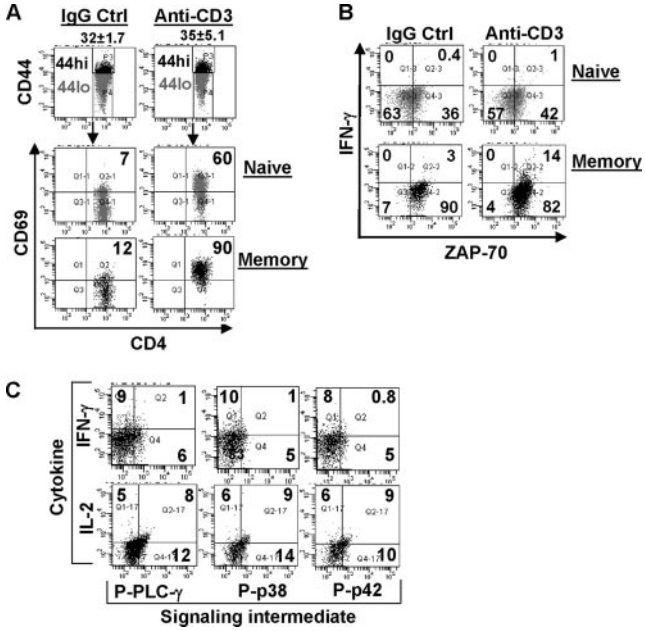

FIGURE 7. In vivo analysis of signaling and function from polyclonal naive and memory CD4 T cells. *A*, Mice ($n = 3$ per group) were injected with anti-CD3 or control IgG and spleens were harvested after 4 h. *First row*, CD44 expression of CD4 T cells in anti-CD3 vs control-treated mice showing delineation and gate for memory (CD44^{high}) and naive (CD44^{low}) T cells. The proportion of CD44^{high}CD4 T cells in control IgG vs anti-CD3-treated mice is indicated as 32 ± 1.7 and 35 ± 5.1 from three mice per group. *Rows 2* and *3*, CD69 expression on naive and memory CD4 T cells in anti-CD3 vs control-treated mice, with the number in the *upper quadrant* indicating percentage of CD69^{high} cells. *B*, Functional coupling of Zap70 vs IFN-γ production gated on naive (CD44^{low}) and memory (CD44^{high}) CD4 T cells in IgG vs anti-CD3-treated mice. Quadrants were drawn based on isotype control and the number in each quadrant represents the percentage of the respective population. Results are representative of three independent experiments. *C*, Correlation of downstream signaling and cytokine production in anti-CD3-stimulated memory CD4 T cells in vivo. Expression of phospho-p38 and phospho-p42 MAPKs and phospho-PLC-γ vs IL-2 or IFN-γ production in memory CD4 T cells isolated from anti-CD3-treated mice. No IFN-γ or IL-2 was observed in cells from control-treated mice and quadrants are drawn based on isotype controls.

effects on IFN-γ production. We transfected fluorescently coupled siRNAs specific for Zap70 or GAPDH into resting memory T cells using nucleofection (see *Materials and Methods*) that we previously found enabled transfection of resting memory T cells (30). Specific silencing of Zap70 led to a reduction in Zap70 expression indicated by a reduction in the MFI of Zap70 in silenced (siRNA$^+$) compared with unsilenced (siRNA$^-$) populations, and Zap70 expression in siRNA$^+$ and siRNA$^-$ control GAPDH transfectants. This siRNA-mediated reduction in Zap70 expression resulted in a 50% reduction in the proportion of memory T cells producing IFN-γ, compared with the higher fraction of IFN-γ producers in siRNA$^-$ and GAPDH siRNA memory T cell transfectants (Fig. 6*C*, *right*). These results demonstrate that Zap70 signaling is essential for both early and late effector function from Ag-recalled memory CD4 T cells, and that a selective reduction of Zap70 expression in memory T cells inhibits their rapid recall.

In vivo coupling of TCR-mediated signaling and function in naive and memory CD4 T cells

Although our results establish differential coupling of function to signaling in memory CD4 T cells stimulated ex vivo, we asked whether similar functional coupling of signaling occurred in vivo

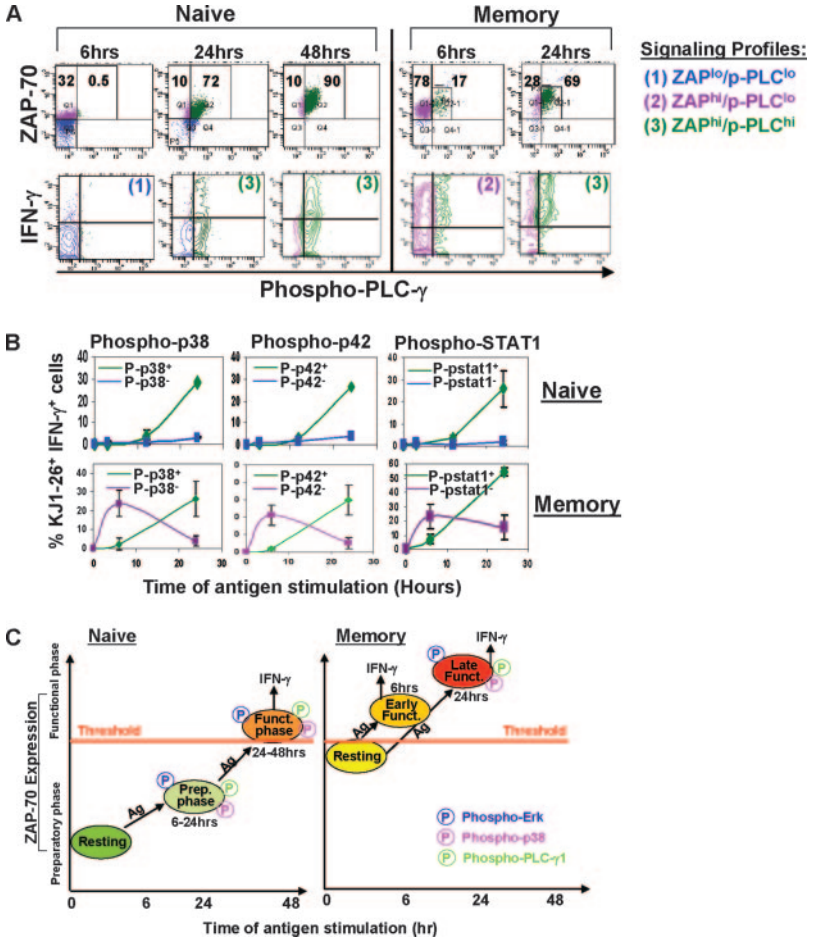

FIGURE 8. Early and late IFN-γ production from memory CD4 T cells are associated with distinct signaling profiles. *A*, Analysis of intracellular phospho-PLCγ (p-PLCγ) vs Zap70 (*top row*), in naive and memory CD4 T cells stimulated with OVA/APC for 6–48 h, gated on live $CD4^+KJ1\text{-}26^+$ cells (*top row*). Three populations based on expression of Zap70 and p-PLCγ are represented by color-coded boxes: 1) $Zap70^{low}pPLC\gamma^{low}$ gate (blue); 2) $Zap70^{high}$/p-PLC^{low}gate (magenta); and 3) $Zap70^{high}pPLC\gamma^{high}$ gate (green). IFN-γ production vs p-PLC-γ from each of these populations is represented by their respective colors in the contour plots (*row 2*). *B*, Graph showing the functional coupling of distal signaling parameters in naive and memory CD4 T cells following antigenic stimulation. IFN-γ production from resting and Ag-stimulated naive and memory CD4 T cells was analyzed in conjunction with intracellular phospho-p38, phospho-p42, and phospho-STAT1, with $IFN\text{-}\gamma^+p\text{-}p38^-$, $IFN\text{-}\gamma^+p\text{-}p42^-$, $IFN\text{-}\gamma^+p\text{-}STAT1^-$ populations represented by magenta; and $IFN\text{-}\gamma^+p\text{-}p38^+$, $IFN\text{-}\gamma^+p\text{-}p42^+$, $IFN\text{-}\gamma^+p\text{-}STAT1^+$ populations represented by green. Both naive and memory CD4 T cells were gated on $CD4^+KJ1\text{-}26^+$ cells. Data are compiled from three independent experiments. *C*, Model for differential coupling of signaling to effector function in memory vs naive CD4 T cells. Naive and memory CD4 T cells are depicted as a function of Zap70 expression (*y*-axis for both plots) vs the time of Ag stimulation (*x*-axis). For naive T cells, Ag activation leads first to a "preparatory phase" characterized by increased Zap70 expression and downstream phosphorylation, followed by a "functional phase" marked by elevated Zap70 expression, and phosphorylation. Resting memory CD4 T cells exhibit elevated Zap70 expression and rapidly progress to an early functional phase ("Early Funct."), with sustained activation leading to a late functional phase ("Late funct.") with a signaling profile similar to that of naive T cells.

in polyclonal memory CD4 T cells. To establish a system for analysis of in vivo signaling and function, we administered low-dose anti-CD3 Ab to unmanipulated BALB/c mice that is known to promote rapid TCR/CD3-triggered in vivo responses (22, 31), and recovered splenocytes after 4 h from anti-CD3 and control Ig-treated mice. The percentages of memory ($CD44^{high}$) and naive ($CD44^{low}$) phenotype CD4 T cells were comparable in mice injected either with anti-CD3 or control IgG (Fig. 7*A*, *top*), indicating that the short-term treatment did not alter the overall proportion of these subsets. Both naive and memory CD4 T cells exhibited up-regulation of the early activation marker CD69 following anti-CD3 stimulation, with a higher proportion of memory CD4 T cells being $CD69^{high}$ (Fig. 7*A*), indicating that in vivo anti-CD3 treatment triggered TCR signaling in both subsets. Analysis of Zap70 and IFN-γ production from naive and memory CD4 T cells in vivo revealed that $CD44^{high}$ memory T cells exhibiting a high level of Zap70 expression mediated rapid IFN-γ production in response to anti-CD3 in vivo (but not control IgG), whereas naive CD4 T cells expressing a lower level of Zap70 did not secrete IFN-γ (Fig. 7*B*), similar to our ex vivo stimulation results. These results establish that rapid effector function occurs exclusively from $Zap70^{high}$ cells in vivo.

We hypothesized that the coordinate analysis of signaling events and cytokine production as performed with Zap70 and IFN-γ could be informative for assessing correlations between downstream signaling events and function. To test this approach, we analyzed the phosphorylation of downstream mediators including PLC-γ, p38 MAPK, and the p42 erk kinase in conjunction with IFN-γ and IL-2 production from in vivo anti-CD3-stimulated memory CD4 T cells as above (in vivo-activated naive CD4 T cells did not produce measurable IFN-γ or IL-2 after 4 h). We found that while a small proportion of memory CD4 T cells exhibited phosphorylated PLC-γ, -p38, and -p42 after short-term anti-CD3 stimulation (Fig. 7*C*), the majority of rapid IFN-γ production

occurred from memory cells that did not exhibit these downstream phosphorylations (Fig. 7*C*, *top row*). By contrast, IL-2 production from memory CD4 T cells was preferentially observed from the subset that up-regulated phosphorylated PLC-γ, p38, and p42 (Fig. 7*C*, *bottom row*), consistent with their known signaling requirement for IL-2 production in primary T cells and T cell lines (32, 33). These results suggest that simultaneous assessment of phosphorylation events and function, while measured at later time points compared with conventional biochemical analyses, may be useful for revealing important associations between signaling events and functional output.

Quantitative differences in distal signaling mark early and late IFN-γ production

We next applied the analysis of downstream signaling and function to determine whether increased Zap70 expression and rapid IFN-γ production in memory T cells was associated with alterations in downstream signaling. We activated OVA-specific naive and memory CD4 T cells with Ag over a broad kinetic window and examined the expression of phosphorylated distal signaling intermediates (p-PLC-γ, phospho-p42, and -p38 MAPKs and p-STAT1) in conjunction with Zap70 protein expression and IFN-γ production.

We found qualitative and kinetic differences in signaling profiles linked to IFN-γ production in naive and memory CD4 T cells (Fig. 8, *A* and *B*). A representative analysis of Zap70, PLC-γ, and IFN-γ demonstrates an accumulation of downstream PLC-γ1 phosphorylation following similar kinetics in naive and memory CD4 T cells, with 24 h of Ag stimulation resulting in a majority of p-PLC-γ^+ cells (Fig. 8*A*, *first row*). Importantly, Zap70 expression and phosphorylated-PLC-γ delineate three distinct signaling profiles in naive and memory subsets (Fig. 8*A*, *top*): 1) Zap70^{low}/p-PLC-γ^{low} (blue) as in resting and 6-h stimulated naive CD4 T cells, 2) Zap70^{high}/p-PLC-γ^{low} (magenta), as in resting and 6 h-stimulated memory CD4 T cells, and 3) Zap70^{high}/p-PLC-γ^{high} (green) found naive and memory CD4 T cells stimulated for ≥24 h. IFN-γ production associated with each of these signaling profiles in naive and memory CD4 T cells is shown in the colored contour plots (Fig. 8*A*, *bottom row*). For naive T cells, the IFN-γ-producing population emerging after 24–48 h derives exclusively from the Zap70^{high}/p-PLC-γ^{high} subset (Fig. 8*A*, *lower left*). By contrast, IFN-γ-producing memory CD4 T cells bore two distinct signaling profiles at early and late times of Ag stimulation; after 6-h stimulation, the IFN-γ^+ population derived predominantly from the Zap70^{high}/p-PLC-γ^{low} subset (Fig. 8*A*, *lower row*), as also observed in vivo, whereas after 24 h of stimulation, IFN-γ producers derived from the Zap70^{high}/p-PLC-γ^{high} subset (Fig. 8*A*, *lower row*, *last plot*).

Similar to our results with p-PLC-γ, we found an accumulation of distal phosphorylated MAPKs (p38 and p42) and STAT1 at late times after activation (≥24 h for naive and memory cells), also consistent with previous biochemical results showing increased phosphorylation after sustained T cell activation (11, 34, 35), with negligible accumulation after 6 h of stimulation in either naive or memory CD4 T cells (data not shown). IFN-γ production from activated naive CD4 T cells occurred exclusively from cells with phosphorylated distal intermediates (Fig. 8*B*, *top row*) together with increased Zap70. By contrast, early IFN-γ production from Ag-stimulated memory CD4 T cells derived from Zap70^{high} cells lacking p-p38, p-p42, or pSTAT1 (Fig. 8*B*, *lower*, magenta line), whereas late IFN-γ production from memory CD4 T cells occurred from cells that had accumulated phosphorylated intermediates, similar to naive cells (Fig. 8*B*, green lines). When taken together, these results demonstrate that rapid IFN-γ by memory CD4 T cells occurs from cells that do not exhibit sustained increases in downstream phosphorylation events, contrasting the extensive accumulation of phosphorylation accompanying late IFN-γ production from stimulation of naive or memory CD4 T cells.

Discussion

The rapid recall response elicited by memory T cells is a hallmark of immunological memory; however, the underlying mechanism(s) for this efficacious response have been unresolved. In this study, we present a novel analysis of TCR-coupled signaling and function in resting and Ag-stimulated naive and memory CD4 T cells, and reveal distinct signaling pathways linked to effector function in these subsets. Specifically, we identify a striking elevation in expression of the Zap70 protein tyrosine kinase in Ag-specific and polyclonal memory compared with naive CD4 T cells. High-level Zap70 expression in memory CD4 T cells was maintained independent of protein synthesis, and was required for their rapid recall function. In vitro results with our Ag-specific system (Fig. 8) together with in vivo analysis of polyclonal naive and memory CD4 T cells (Fig. 7) indicate that rapid IFN-γ from memory CD4 T cells occurs from Zap70^{high} cells in the context of fewer downstream signaling events compared with IFN-γ production resulting from sustained activation of naive CD4 T cells. Together, our results reveal a unique biochemical signature of memory CD4 T cells suggesting that increased Zap70 and its association with the TCR/CD3 complex may initiate more efficient signaling from TCR ligation to enhanced effector function.

We demonstrate here elevated expression of the Zap70 protein in both Ag-specific and polyclonal mouse memory CD4 T cells as an inherent property of T cell memory. The greatest difference in Zap70 expression (>5-fold) occurred between pure naive DO11.10 TCR cells and memory CD4 T cells in the mouse. Polyclonal naive (CD44^{low}) T cells in adult mice showed levels of Zap70 slightly greater than the pure naive populations, suggesting that levels of Zap70 may also be affected by homeostatic turnover of these cells in the periphery (36) or during aging (37). The comparable transcript level of Zap70 in resting naive and memory CD4 T cells coupled with our findings that elevated Zap70 expression in memory CD4 T cells can be maintained independent of new protein synthesis together indicate increased stability and/or reduced turnover of the Zap70 protein in memory T cells. Zap70 protein stability in NK cells and lymphomas has been linked to both ubiquitin-mediated regulation and association to molecular chaperones (38, 39), and these mechanisms could also be operable in T cells. The increased stability of specific proteins in memory CD4 T cells may reflect an overall change in cell physiology that contribute to their enhanced functions, lifespan, and turnover.

Our results demonstrate that elevated Zap70 expression can act as a biochemical indicator for effector capacity, as IFN-γ-production was exclusively from T cells that had up-regulated Zap70 expression in CD4 T cells. Moreover, we show that elevated Zap70 expression is required for rapid IFN-γ from memory CD4 T cells, as early IFN-γ production was inhibited when Zap70 expression was selectively down-modulated using siRNA, and completely blocked when Zap70 expression and signaling were prevented by the inhibitor piceatannol. Although Zap70 signaling has been linked to downstream processes such as PLC-γ activation and calcium flux that lead to IL-2 production (40, 41), our findings indicate that Zap70 is a critical proximal transducer for effector function and a key regulator of recall function in memory CD4 T cells.

The increased protein expression of Zap70 appeared to be more central in regulation of memory T cell function, and we found only modest up-regulation of Zap70 phosphorylation at residue Y319

(shown to be important in TCR signaling (26) in memory T cells at earlier time points (30 min–6 h) that did not correlate with function. These findings are consistent with our previous results of low phospho-Zap70 content in lysates of phenotypic memory CD4 T cells (10); however, we cannot rule out transient phosphorylation events (42), or other regulatory phosphorylation sites playing a role (43). Our finding of increased Zap70 associated to the TCR/CD3 complex also suggests that signaling efficiency may likewise be controlled via these increased proximal associations as previously suggested (44, 45).

The majority of studies of TCR-mediated signaling examine intracellular events seconds to minutes following TCR ligation. By contrast, we demonstrate that sustained activation of T cells leads to profound changes in the expression and phosphorylation state of TCR-coupled signaling intermediates, and these changes are differentially associated with distinct T cell functions. We propose a signaling threshold model in which the level of Zap70 controls effector responses in CD4 T cells, with increased Zap70 expression leading to more efficient cellular responses (Fig. 8*C*). In naive CD4 T cells, Zap70 expression is low accounting for the lack of effector function; however, sustained antigenic stimulation (6–24 h) leads to increased Zap70 expression requiring new transcription and translational (Fig. 4*B* and data not shown) and also an accumulation of downstream phosphorylation events comprising a preparatory phase with low functional output (Fig. 8*C*). When the minimum threshold level of Zap70 expression is achieved, cells enter into a functional phase, leading to IFN-γ production. For memory CD4 T cells, the level of Zap70 in the resting state is already at this threshold level, hence the preparatory phase is bypassed and cells enter directly into the early functional phase without the requirement for continual downstream phosphorylation (Fig. 8*C*). Sustained stimulation of memory CD4 T cells results in late effector function marked by further increases in Zap70 expression and the accumulation of phosphorylation events similar to the functional phase of naive T cells, and distinct from the early functional phase.

The involvement of Zap70 expression in setting up a threshold value for signaling has been recently demonstrated in chronic lymphocytic leukemia (46) in which Zap70 up-regulation has been associated with aggressive disease (47). A certain threshold level of Zap70 expression in chronic lymphocytic leukemia cells was shown to enhance IgM signaling, and exceeding this threshold did not further enhance IgM signaling or downstream adaptor/signaling events (46). These studies suggest that levels of Zap70 kinase may serve as a generalized proximal signaling indicator for downstream functions in lymphocytes. Additional alterations associated with memory T cell signaling previously identified such as increased membrane rafts (48), more rapid immune synapse formation (49) and epigenetic changes in cytokine loci (48) may synergize with the increased proximal signals to facilitate rapid recall responses.

In conclusion, our results provide novel biochemical insights into the rapid recall of memory T cells using a multiparameter approach, identifying elevated expression of the proximal Zap70 kinase and quantitative differences in downstream phosphorylation as critical elements that distinguish the memory pathway to efficacious responses.

Acknowledgments

We thank Dr. Zachary Roberts for help with the PCR assays and analysis, Dr. Paul Luther for guidance with confocal microscopy, Smita Chandran for help with in vivo experiments, and Wendy Lai for mouse colony maintenance. We also thank Drs. Arthur Weiss, George Tsokos, and Jan Cerny for critical reading of this manuscript and Dr. Mark Williams for helpful discussions.

Disclosures

The authors have no financial conflict of interest.

References

1. Kaech, S. M., E. J. Wherry, and R. Ahmed. 2002. Effector and memory T-cell differentiation: implications for vaccine development. *Nat. Rev. Immunol.* 2: 251–262.
2. Dubey, C., M. Croft, and S. L. Swain. 1996. Naive and effector CD4 T cells differ in their requirements for T cell receptor versus costimulatory signals. *J. Immunol.* 157: 3280–3289.
3. Rogers, P. R., C. Dubey, and S. L. Swain. 2000. Qualitative changes accompany memory T cell generation: faster, more effective responses at lower doses of antigen. *J. Immunol.* 164: 2338–2346.
4. Kane, L. P., J. Lin, and A. Weiss. 2000. Signal transduction by the TCR for antigen. *Curr. Opin. Immunol.* 12: 242–249.
5. Chan, A. C., M. Iwashima, C. W. Turck, and A. Weiss. 1992. ZAP-70: A 70 kd protein-tyrosine kinase that associates with the TCR ζ chain. *Cell* 71: 649–662.
6. Iwashima, M., B. A. Irving, N. S. van Oers, A. C. Chan, and A. Weiss. 1994. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science* 263: 1136–1139.
7. Jordan, M. S., A. L. Singer, and G. A. Koretzky. 2003. Adaptors as central mediators of signal transduction in immune cells. *Nat. Immunol.* 4: 110–116.
8. Chandok, M. R., and D. L. Farber. 2004. Signaling control of memory T cell generation and function. *Semin. Immunol.* 16: 285–293.
9. Farber, D. L., M. Luqman, O. Acuto, and K. Bottomly. 1995. Control of memory CD4 T cell activation: MHC class II molecules on antigen presenting cells and CD4 ligation inhibit memory but not naive CD4 T cells. *Immunity* 2: 249–259.
10. Farber, D. L., O. Acuto, and K. Bottomly. 1997. Differential T cell receptor-mediated signaling in naive and memory CD4 T cells. *Eur. J. Immunol.* 27: 2094–2101.
11. Hussain, S. F., C. F. Anderson, and D. L. Farber. 2002. Differential SLP-76 expression and TCR-mediated signaling in effector and memory CD4 T cells. *J. Immunol.* 168: 1557–1565.
12. Perez, O. D., and G. P. Nolan. 2002. Simultaneous measurement of multiple active kinase states using polychromatic flow cytometry. *Nat. Biotechnol.* 20: 155–162.
13. Krutzik, P. O., M. R. Clutter, and G. P. Nolan. 2005. Coordinate analysis of murine immune cell surface markers and intracellular phosphoproteins by flow cytometry. *J. Immunol.* 175: 2357–2365.
14. Murphy, K. M., A. B. Heimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of $CD4^+CD8^+TCR^{lo}$ thymocytes in vivo. *Science* 250: 1720–1723.
15. Shinkai, Y., G. Rathbun, K. P. Lam, E. M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A. M. Stall, et al. 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 68*:* 855–867.
16. Patke, D. S., and D. L. Farber. 2005. Modulation of memory CD4 T cell function and survival potential by altering the strength of the recall stimulus. *J. Immunol.* 174: 5433–5443.
17. Bingaman, A. W., D. S. Patke, V. R. Mane, M. Ahmadzadeh, M. Ndejembi, S. T. Bartlett, and D. L. Farber. 2005. Novel phenotypes and migratory properties distinguish memory CD4 T cell subsets in lymphoid and lung tissue. *Eur. J. Immunol.* 35: 3173–3186.
18. Moulton, V. R., N. D. Bushar, D. B. Leeser, D. S. Patke, and D. L. Farber. 2006. Divergent generation of heterogeneous memory CD4 T cells. *J. Immunol.* 177: 869–876.
19. Ahmadzadeh, M., and D. L. Farber. 2002. Functional plasticity of an antigen-specific memory CD4 T cell population. *Proc. Natl. Acad. Sci. USA* 99: 11802–11807.
20. Patke, D. S., M. Ahmadzadeh, A. W. Bingaman, and D. L. Farber. 2005. Anti-CD3 priming generates heterogeneous antigen-specific memory CD4 T cells. *Clin. Immunol.* 117: 125–132.
21. Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta\ C_T)$ method. *Methods* 25: 402–408.
22. Scott, D. E., W. C. Gause, F. D. Finkelman, and A. D. Steinberg. 1990. Anti-CD3 antibody induces rapid expression of cytokine genes in vivo. *J. Immunol.* 145: 2183–2188.
23. Sabbagh, L., C. C. Srokowski, G. Pulle, L. M. Snell, B. J. Sedgmen, Y. Liu, E. N. Tsitsikov, and T. H. Watts. 2006. A critical role for TNF receptor-associated factor 1 and Bim down-regulation in CD8 memory T cell survival. *Proc. Natl. Acad. Sci. USA* 103: 18703–18708.
24. Ahmadzadeh, M., S. F. Hussain, and D. L. Farber. 2001. Heterogeneity of the memory CD4 T cell response: persisting effectors and resting memory T cells. *J. Immunol.* 166: 926–935.
25. Ndejembi, M. P., J. R. Teijaro, D. S. Patke, A. W. Bingaman, M. R. Chandok, A. Azimzadeh, S. G. Nadler, and D. L. Farber. 2006. Control of memory CD4 T cell recall by the CD28/B7 costimulatory pathway. *J. Immunol.* 177: 7698–7706.
26. Di Bartolo, V., D. Mege, V. Germain, M. Pelosi, E. Dufour, F. Michel, G. Magistrelli, A. Isacchi, and O. Acuto. 1999. Tyrosine 319, a newly identified phosphorylation site of ZAP-70, plays a critical role in T cell antigen receptor signaling. *J. Biol. Chem.* 274: 6285–6294.
27. Sachs, K., O. Perez, D. Pe'er, D. A. Lauffenburger, and G. P. Nolan. 2005. Causal protein-signaling networks derived from multiparameter single-cell data. *Science* 308: 523–529.

28. Soede, R. D., Y. M. Wijnands, I. Van Kouteren-Cobzaru, and E. Roos. 1998. ZAP-70 tyrosine kinase is required for LFA-1-dependent T cell migration. *J. Cell. Biol.* 142: 1371–1379.
29. Hannon, G. J. 2002. RNA interference. *Nature* 418: 244–251.
30. Lai, W., C. H. Chang, and D. L. Farber. 2003. Gene transfection and expression in resting and activated murine CD4 T cell subsets. *J. Immunol. Methods* 282: 93–102.
31. Ellenhorn, J. D., R. Hirsch, J. P. Hartley, and J. A. Bluestone. 1989. Dose-dependent activation of murine T cells following in vivo administration of anti-murine CD3. *Transplant Proc.* 21: 1013–1014.
32. Kane, L. P., J. Lin, and A. Weiss. 2000. Signal transduction by the TCR for antigen. *Curr. Opin. Immunol.* 12: 242–249.
33. Yablonski, D., T. Kadlecek, and A. Weiss. 2001. Identification of a phospholipase C-γ1 (PLC-γ1) SH3 domain-binding site in SLP-76 required for T-cell receptor-mediated activation of PLC-γ1 and NFAT. *Mol. Cell. Biol.* 21: 4208–4218.
34. Ahmadzadeh, M., S. F. Hussain, and D. L. Farber. 1999. Effector CD4 T cells are biochemically distinct from the memory subset: evidence for long-term persistence of effectors in vivo. *J. Immunol.* 163: 3053–3063.
35. Krishnan, S., V. G. Warke, M. P. Nambiar, H. K. Wong, G. C. Tsokos, and D. L. Farber. 2001. Generation and biochemical analysis of human effector CD4 T cells: alterations in tyrosine phosphorylation and loss of CD3ζ expression. *Blood* 97: 3851–3859.
36. Song, K., R. L. Rabin, B. J. Hill, S. C. De Rosa, S. P. Perfetto, H. H. Zhang, J. F. Foley, J. S. Reiner, J. Liu, J. J. Mattapallil, et al. 2005. Characterization of subsets of $CD4^+$ memory T cells reveals early branched pathways of T cell differentiation in humans. *Proc. Natl. Acad. Sci. USA* 102: 7916–7921.
37. Miller, R. A. 2000. Effect of aging on T lymphocyte activation. *Vaccine* 18: 1654–1660.
38. Castro, J. E., C. E. Prada, O. Loria, A. Kamal, L. Chen, F. J. Burrows, and T. J. Kipps. 2005. ZAP-70 is a novel conditional heat shock protein 90 (Hsp90) client: inhibition of Hsp90 leads to ZAP-70 degradation, apoptosis, and impaired signaling in chronic lymphocytic leukemia. *Blood* 106: 2506–2512.
39. Paolini, R., R. Molfetta, M. Piccoli, L. Frati, and A. Santoni. 2001. Ubiquitination and degradation of Syk and ZAP-70 protein tyrosine kinases in human NK cells upon CD16 engagement. *Proc. Natl. Acad. Sci. USA* 98: 9611–9616.
40. Herndon, T. M., X. C. Shan, G. C. Tsokos, and R. L. Wange. 2001. ZAP-70 and SLP-76 regulate protein kinase C-theta and NF-κB activation in response to engagement of CD3 and CD28. *J. Immunol.* 166: 5654–5664.
41. Wange, R. L., N. Isakov, T. R. Burke, Jr., A. Otaka, P. P. Roller, J. D. Watts, R. Aebersold, and L. E. Samelson. 1995. F2(Pmp)2-TAM ζ3, a novel competitive inhibitor of the binding of ZAP-70 to the T cell antigen receptor, blocks early T cell signaling. *J. Biol. Chem.* 270: 944–948.
42. Yokosuka, T., K. Sakata-Sogawa, W. Kobayashi, M. Hiroshima, A. Hashimoto-Tane, M. Tokunaga, M. L. Dustin, and T. Saito. 2005. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. *Nat. Immunol.* 6: 1253–1262.
43. Zhao, Q., B. L. Williams, R. T. Abraham, and A. Weiss. 1999. Interdomain B in ZAP-70 regulates but is not required for ZAP-70 signaling function in lymphocytes. *Mol. Cell. Biol.* 19: 948–956.
44. Duplay, P., M. Thome, F. Herve, and O. Acuto. 1994. $p56^{lck}$ interacts via its src homology 2 domain with the ZAP-70 kinase. *J. Exp. Med.* 179: 1163–1172.
45. Thome, M., P. Duplay, M. Guttinger, and O. Acuto. 1995. Syk and ZAP-70 mediate recruitment of $p56^{lck}$/CD4 to the activated T cell receptor/CD3/ζ complex. *J. Exp. Med.* 181: 1997–2006.
46. Chen, L., J. Apgar, L. Huynh, F. Dicker, T. Giago-McGahan, L. Rassenti, A. Weiss, and T. J. Kipps. 2005. ZAP-70 directly enhances IgM signaling in chronic lymphocytic leukemia. *Blood* 105: 2036–2041.
47. Rassenti, L. Z., L. Huynh, T. L. Toy, L. Chen, M. J. Keating, J. G. Gribben, D. S. Neuberg, I. W. Flinn, K. R. Rai, J. C. Byrd, et al. 2004. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. *N. Engl. J. Med.* 351: 893–901.
48. Northrop, J. K., R. M. Thomas, A. D. Wells, and H. Shen. 2006. Epigenetic remodeling of the IL-2 and IFN-γ loci in memory CD8 T cells is influenced by CD4 T cells. *J. Immunol.* 177: 1062–1069.