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*J Immunol* 2013; 190:3089-3099; Prepublished online 20 February 2013; doi: 10.4049/jimmunol.1202408

http://www.jimmunol.org/content/190/7/3089

Supplementary Material [http://www.jimmunol.org/content/suppl/2013/02/20/jimmunol.1202408.DC1](http://www.jimmunol.org/content/suppl/2013/02/20/jimmunol.1202408.DC1)

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Differential Polarization of C-Terminal Src Kinase between Naive and Antigen-Experienced CD8+ T Cells

Jessica G. Borger,* Andrew Filby,† and Rose Zamoyska*

In CD8+ T cells, engagement of the TCR with agonist peptide:MHC molecules causes dynamic redistribution of surface molecules including the CD8 coreceptor to the immunological synapse. CD8 associates with the Src-family kinase (SFK) Lck, which, in turn, initiates the rapid tyrosine phosphorylation events that drive cellular activation. Compared with naive T cells, Ag-experienced CD8+ T cells make shorter contacts with APC, are less dependent on costimulation, and are triggered by lower concentrations of Ag, yet the molecular basis of this more efficient response of memory T cells is not fully understood. In this article, we show differences between naive and Ag-experienced CD8+ T cells in colocalization of the SFKs and their negative regulator, C-terminal Src kinase (Csk). In naive CD8+ T cells, there was pronounced colocalization of SFKs and Csk at the site of TCR triggering, whereas in Ag-experienced cells, Csk displayed a bipolar distribution with a proportion of the molecules sequestered within a cytosolic area in the distal pole of the cell. The data show that there is differential redistribution of a key negative regulator away from the site of TCR engagement in Ag-experienced CD8+ T cells, which might be associated with the more efficient responses of these cells on re-exposure to Ag. The Journal of Immunology, 2013, 190: 3089–3099.

A hallmark of immunological memory is that on Ag re-encounter, previously activated T cells have a faster response than naive T cells. Memory CD8+ T cells may be activated by peptide concentrations up to 50 times lower than those required to activate naive CD8+ T cells (1) and show a shorter lag time for entry into cell cycle and production of cytokines (2). However, the molecular basis of the increased sensitivity of memory T cells remains poorly understood. Activation of αβ CD8+ T cells requires binding of the TCR together with the coreceptor, CD8, to MHC class I molecules containing antigenic peptides (pMHC), resulting in the redistribution of these molecules to a discrete area called the immunological synapse (IS). The CD8 coreceptor is directly associated with a member of the Src-family kinases (SFK), p56lck (Lck) (3), which is important for the coreceptor function of CD8 (4). The immediate targets of Lck are the ITAMs of the TCR-associated CD3 molecule and ζ-chains and ZAP70. In resting T cells, the activity of the SFKs is regulated by the balancing interactions of the phosphatase, CD45 (5, 6), and the kinase, C-terminal Src kinase (Csk) (7, 8). Precisely how signal transduction is initiated is unclear, but it involves a combination of colocalization of the TCR and its coreceptor, and possible further oligomerization of these complexes, which increases the local concentrations of kinases and their substrates (9). In addition, the active exclusion of negative regulators of signaling, such as phosphatases, particularly CD45 (10) and Csk from the IS, is thought to play an important role in signal initiation (11). Indeed, resting T cells are maintained in a quiescent state by the local action of phosphatases, as shown by the ability of phosphatase inhibitors to activate T cells in the absence of receptor engagement (12).

One possible explanation for the increased sensitivity of effector/memory CD8+ T cells to antigenic stimulation is that the balance or distribution of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) is regulated differently than in naive cells, resulting in their characteristic lower activation threshold. The availability of Lck is a key determinant of the activation threshold of primary T cells (13, 14); therefore, how Csk regulates the activity of Lck has an important influence on T cell activation (15, 16). Csk is a cytoplasmic PTK that downregulates SFK activity by phosphorylating the C-terminal inhibitory tyrosine and promoting a closed, inactive conformation of the kinase (17). The activity of Csk is regulated by its cellular localization (15, 16, 18) and by interactions via its SH2 domain, which increases the kinase activity of Csk (15, 19). Csk is recruited to the plasma membrane (PM), and thus the vicinity of its SFK target proteins via binding of its SH2 domain to phosphorylated transmembrane adapter proteins such as Csk-binding protein/phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG) (20, 21). The second major SFK expressed in T cells, p59fyn (Fyn), plays a role in the recruitment of Csk to the PM by phosphorylating PAG after TCR activation. Fyn selectively phosphorylates PAG on Y314, which is the primary residue that interacts with the Csk SH2 domain (20). Together these enzymes modulate signal duration downstream of the TCR, forming an integral part of a negative feedback system that regulates T cell activation. Overexpression of Csk resulted in inhibition of TCR-induced Tyr phosphorylation and IL-2 production (8). Similarly, constitutively targeting Csk to the PM inhibited T cell activation (15, 16), emphasizing the
importance of cellular localization for Csk function. Furthermore, loss of PAGY314 in Fyn knockout (FynKO) CD8+ T cells decreased the duration of pMHC1 stimulation required to stimulate IL-2 production, suggesting that Fyn is involved in a negative feedback loop involving Csk (22).

Early localization of TCR signaling mediators after TCR engagement has been well characterized in CD8+ cytotoxic effector cells (CTL) (23). Positive regulators of TCR signaling, such as Lck and Zap-70, were shown to concentrate at the site of contact within 5–10 min of interaction of the TCR with pMHC1 on the APC. However, little is known about the localization of negative regulators in CD8+ T cells. Furthermore, relatively few studies using naive or memory CD8+ T cells have addressed the localization of TCR signaling mediators at a single-cell level. Biochemical analysis showed that early after T cell activation, PAGY314 and Csk colocalized at the PM, which correlated with increased phosphorylated Lck on its inhibitory residue Y505 (24, 25). However, it was unclear where these interactions occurred relative to the site of T cell–APC contact or how these proteins were distributed. In this study, we asked whether there was differential localization of key signaling mediators between naive and Ag-experienced CD8+ T cells that could account for the differences in their kinetics of response.

Using four-color confocal microscopy and an imaging flow cytometer, we followed the redistribution of the TCR and CD8 coreceptor together with critical FAKs during early TCR engagement. We found that Lck was more efficiently recruited to the site of TCR engagement in Ag-experienced rather than naive CD8+ T cells. Moreover, in Ag-experienced CD8+ T cells, Csk and Fyn shared a bipolar distribution that was distinct from that found in naive cells. In both cell types, Csk and Fyn colocalized with LckY305 at the site of TCR engagement, but in Ag-experienced CD8+ T cells, a distinct pool of Csk and Fyn concentrated within the cytosol at the distal pole of the cell. Our results suggest that altered and more efficient signal transduction intrinsic to Ag-experienced CD8+ T cells may be due, in part, to the spatial reorganization of critical T cell signaling mediators.

Materials and Methods

Mice

F5 Rag-1−/− mice, transgenic for a class I MHC–restricted TCR with a cognate peptide Ag derived from an influenza virus nucleoprotein, NP68, have been described previously (26). The generation of F5 FynKO mice have previously been described (22). Mice were bred in the specific pathogen-free facility at the University of Edinburgh (U.K.). All experiments were approved under a Project License granted by the Home Office (U.K.) and conducted in accordance with the institutional and ethical guidelines of the University of Edinburgh.

Mouse T cell primary cultures and T cell stimulation

Cell preparation and in vitro generation of Ag-experienced CD8+ T cells. Single-cell suspensions from total lymph node (LN) and spleen of mice were cultured in IMDM medium supplemented with 5% FCS, l-glutamine, 100 U/ml penicillin and streptomycin (Life Technologies), and 50 mg/ml streptomycin (PeproTech, London, U.K.) and 10 ng/ml IL-15 (all from PeproTech, London, U.K.) and conducted in accordance with the institutional and ethical guidelines of the University of Edinburgh.

Cell preparation for confocal and ImageStream analysis. CD8+ T cells were preincubated with a mixture of 10 μg/ml biotinylated anti-TCRβ (H57-597; eBiosciences) and anti-CD8α (KT15; Abcam) Abs on ice for 30 min. Postwashing in 1× PBS, cross-linking was achieved by addition of 5 μg/ml streptavidin-543 on ice for 10 min. Cells were then incubated at 4˚C for cell activation by addition of 4% PFA.

Naive F5 LN cells or Ag-experienced CD8+ T cells were incubated in 96-well tissue culture plates (Nunc, Penfield, NY) at a density of 2.5 × 10^5 cells/well in complete media containing soluble peptide at concentrations of 10−6–10−11 M NP68 or 10−7 M control peptide from the GAG protein of the SF2 strain of HIV (aa 390–398). Alternately, cells were incubated for 30 min on ice with biotin-labeled TCR (H57-597; eBiosciences) and biotin-labeled CD8 (KT15; Abcam) at 10 μg/ml, washed once, and then seeded into 96-well tissue culture plates containing complete media supplemented with streptavadin Alexa Fluor (AF) 405 at 1/1000 (Molecular Probes, Invitrogen, Paisley, U.K.). To inhibit phosphatases, we added 100 μM pervanadate for 20 min at 37˚C before downstream applications.

Cell stimulation. For confocal and ImageStream analysis, CD8+ T cells were preincubated with a mixture of 10 μg/ml biotinylated anti-TCRβ (H57-597; eBiosciences) and anti-CD8α (KT15; Abcam) Abs on ice for 30 min. Postwashing in 1× PBS, cross-linking was achieved by addition of 5 μg/ml streptavidin-543 on ice for 10 min. Cells were then incubated at 4˚C for cell activation by addition of 4% PFA.

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and red (120 mW) laser excitation turned on. The system was ASSIST calibrated immediately before acquisition (28). Single stained controls were collected with bright-field illumination turned off to generate a compensation matrix postacquisition using the wizard imbedded within the IDEAS analysis software package (Amnis; an example matrix is included in Supplemental Fig. 2). TCR/CD8 AF450 emission light was collected in camera channel 1, SFK AF488 in channel 2, and CSK AF647 in channel 5. Bright-field illumination was set in channel 4, and a cell classifier of 20 was set on the area of the bright filed image to exclude debris from the data file. A minimum of 10,000 single, live cells was collected per sample at 40× magnification. Raw image files (rif) were compensated using the defined matrix values, and the gating analysis strategy set out in Supplemental Fig. 2 was implemented across all samples to identify live, single in focus AF450, AF488, and AF647 triple-positive cells. A Pearson correlation coefficient-based feature within the IDEAS analysis software called “Bright Detail Similarity (BDS) R3” was used to determine the pairwise colocalization of the signals on a per-cell basis (29).

Results

TCR plus CD8 engagement optimally redistributes Lck to the site of activation in naive and Ag-experienced CD8+ T cells

To compare naive and in vitro–generated Ag-experienced CD8+ T cells, we used Rag−/− F5 TCR transgenic mice, in which all CD8+ T cells recognize NP68 peptide presented by H-2Db (26), providing a homogenous population of CD8+ T cells. Naive CD8+ T cells were obtained from peripheral LN, whereas Ag-experienced cells were generated in vitro by stimulation with peptide for 3 d followed by 4-d incubation in IL-2– and IL-15–supplemented medium. We confirmed that Ag-experienced F5 T cells were more sensitive to stimulation than naive F5 T cells by measuring TCR downregulation and Erk phosphorylation after stimulation with either peptide or Ab-mediated cross-linking (Supplemental Fig. 1). Lower doses of peptide were required to downregulate TCR (Supplemental Fig. 1A), whereas phospho-Erk was observed with faster kinetics and in more cells in the Ag-experienced population (Supplemental Fig. 1B), confirming that they were more sensitive to stimulation than naive T cells, as described previously (1).

To investigate whether the heightened responses of Ag-experienced CD8+ T cells to TCR stimulation could be caused by differences in the distribution of key signaling mediators between naive and Ag-experienced cells, we asked how the distribution and activation of Lck was influenced by engagement of the TCR or coreceptor, or both. Cross-linking Abs were used to stimulate T cells to follow redistribution of molecules to defined stimuli in the absence of APC and additional costimulatory or accessory molecules. We addressed the efficiency of mAb cross-linking to CD3e or TCRβ alone, or the combination of TCRβ plus CD8α, and measured Lck and phosphorylated Tyr (p-Tyr) residues by confocal microscopy. Cross-linking for 5 min with CD3e alone, TCRβ alone, or TCRβ plus CD8α drove discrete capping in both naive and Ag-experienced CD8+ T cells as expected (Fig. 1). In naive CD8+ T cells, cross-linking CD3e alone caused only a small proportion of cells (6%) to redistribute Lck to the CD3e cap (Table I). In contrast, cross-linking with TCRβ Ab alone caused more cells (20%) to redistribute Lck (Fig. 1A, Table I). The strongest colocalization of Lck with capped TCR occurred after TCRβ cross-linking with CD8α, whereupon 28% of cells showed redistribution of Lck to the cap (Fig. 1A, Table I). Similarly, p-Tyr recruitment to the cap site occurred in more cells after TCRβ and TCRβ/CD8α cross-linking and considerably fewer after cross-linking of CD3e alone (Fig. 1C, Table I), despite the latter generally being considered to be a better stimulus for T cell activation. Ag-experienced CD8+ T cells behaved similarly to naive T cells, although cells showed tighter colocalization of Lck and p-Tyr residues to the cap site for all the stimuli (Fig. 1B, 1D, Table I). In regard to cross-linking of TCRβ and TCRβ/CD8α coligation, there was a 2-fold increase in the number of cells that cocapped Lck in Ag-experienced compared with naive CD8+ T cells, a trend seen also in p-Tyr localization (Table I). Clearly for both naive and Ag-experienced CD8+ T cells, direct engagement of the coreceptor with TCR optimized recruitment of Lck to the site of capping, although this was improved in Ag-experienced cells.

Differential distribution of Csk and Fyn between naive and Ag-experienced CD8+ T cells

We asked whether other upstream positive and negative regulators of TCR signals were differentially distributed upon TCR triggering in naive versus Ag-experienced CD8+ T cells. Lck is the most proximal kinase to be activated upon TCR activation, with recent literature suggesting that there is dynamic regulation of Lck activity already at the basal state, partly because of the localization...
of Csk, which determines TCR signaling initiation and sensitivity (16, 30). Csk is critical for downregulating Lck signals because it is the sole kinase able to phosphorylate the SFK regulatory Tyr, Lck\(^{Y505}\). In the absence of Csk (31) or upon mutation of Lck\(^{Y505}\) to alanine (Ala) (32), TCR signaling is dysregulated, and after activation, T cell signal termination is impaired. Fyn has also been implicated in negative regulation of T cell signaling as it phosphorylates Y314 on the transmembrane adapter PAG facilitating recruitment of cytosolic Csk to the PM. In the absence of Fyn, T cell responses are slower to turn off and can predispose to autoimmunity (33). Naïve CD8\(^+\) T cells, in a resting state, display a homogeneous distribution of p-Tyr, Lck, Lck\(^{Y505}\), and Fyn at the cell periphery identified by TCR\(\beta\) and CD8\(\alpha\) staining (Fig. 1). Despite lacking the N-terminal modifications of palmitoylation, myristoylation, and S-acetylation that direct Lck and Fyn to the PM (34–36), Csk was also observed to localize with these two SFK members. The average Pearson correlation coefficient indicated strong colocalization of Csk with both Lck and Fyn (Rr = 0.74, 0.78, respectively; Fig. 2A). Because Csk is the only kinase to phosphorylate Lck\(^{Y505}\) (7, 24), the relative distribution of Csk and Lck\(^{Y505}\) was investigated. In naïve CD8\(^+\) T cells, Lck\(^{Y505}\) was homogenously distributed at the cell periphery, highly colocalized with Csk (Rr = 0.81; Fig. 2A).

In Ag-experienced CD8\(^+\) T cells, basal levels of p-Tyr were again detected at the cell periphery together with Lck (Fig. 2B). Strikingly, in contrast with naïve cells, Csk distributed not only with SFKs at the cell periphery of Ag-experienced CD8\(^+\) T cells, and in the latter population compared with naive CD8 T cells (Supplemental Fig. 1).

**Table I. Efficiency of Ab cross-linking in redistributing Lck and pY**

<table>
<thead>
<tr>
<th>Capping Ab</th>
<th>Cocapping Ab</th>
<th>T Cell Subset</th>
<th>No. of Cells Counted</th>
<th>Cells Capped (%)</th>
<th>Cells Cocapped (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CD3e</td>
<td>Lck</td>
<td>Naive</td>
<td>1260</td>
<td>27</td>
<td>6.1</td>
</tr>
<tr>
<td>pY</td>
<td>Ag experienced</td>
<td>1385</td>
<td>16</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Naive</td>
<td>1260</td>
<td>27</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Anti-TCR(\beta)</td>
<td>Lck</td>
<td>Naive</td>
<td>1637</td>
<td>32</td>
<td>20</td>
</tr>
<tr>
<td>pY</td>
<td>Ag experienced</td>
<td>760</td>
<td>34</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Naive</td>
<td>1637</td>
<td>32</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Anti-TCR(\beta)/CD8(\alpha)</td>
<td>Lck</td>
<td>Naive</td>
<td>1325</td>
<td>20</td>
<td>28</td>
</tr>
<tr>
<td>pY</td>
<td>Ag experienced</td>
<td>826</td>
<td>23</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Naive</td>
<td>1325</td>
<td>20</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ag experienced</td>
<td>826</td>
<td>23</td>
<td>62</td>
<td></td>
</tr>
</tbody>
</table>

Velocity software was used to manually count individual Ab cross-linked naive and Ag-experienced CD8\(^+\) T cells and count the number of cells cocapping Lck and pY.

Bipolar distribution of Csk and Fyn upon TCR engagement of Ag-experienced, but not naïve CD8\(^+\) T cells

As Csk and Fyn are regulators of TCR signaling and having identified a second intracellular pool of colocalized Csk and Fyn in Ag-experienced cells that was not present in naïve CD8\(^+\) T cells, we asked how Csk and Fyn behaved upon TCR stimulation in both cell types. After TCR\(\beta\)/CD8\(\alpha\) cross-linking, naïve T cells showed strong association of Csk with p-Tyr, Lck, Lck\(^{Y505}\), and Fyn (Rr = 0.76, 0.74, 0.74, and 0.82, respectively; Fig. 3A). These associations occurred at the site of capping only. In contrast, Ag-experienced CD8\(^+\) T cells showed much lower association of Csk with p-Tyr, Lck, and Lck\(^{Y505}\) (Rr = 0.56, 0.57, and 0.47, respectively), whereas retaining a strong association between Csk and Fyn (Rr = 0.77; Fig. 3B).

The reason that Csk was less well associated with Lck and p-Tyr in Ag-experienced CD8\(^+\) T cells after stimulation was because the cytoplasmic pool of Csk, which we identified in resting Ag-experienced cells (Fig. 2B), remained in the area distal to the TCR\(\beta\)/CD8\(\alpha\) complex even after TCR cross-linking. As a consequence, although the capped TCR remained as, or even more, tightly associated with p-Tyr, Lck, and Lck\(^{Y505}\), the association between these TCR\(\beta\)/CD8\(\alpha\) and Csk reduced from Rr = 0.51 in naïve T cells to Rr = 0.41 in Ag-experienced cells (Fig. 3C). As before, Fyn retained stronger colocalization with Csk than with capped TCR in both naïve and Ag-experienced CD8\(^+\) T cells, and in the latter was seen in the distal cytoplasmic area.

To visualize better the proportion of molecules that redistributed toward the proximal and distal poles of stimulated naïve and Ag-experienced CD8\(^+\) T cells, we used spectral overlaps and RGB histogram analysis. A merge image sectioned from the proximal (designated by an asterisk) to the opposing, distal end (trajectory represented by a white line) showed that in naïve CD8\(^+\) T cells, the majority of Lck, Lck\(^{Y505}\), Fyn, and Csk molecules polarized to the site of the TCR\(\beta\)/CD8\(\alpha\) cap (Fig. 4A). Similar analysis of Ag-experienced CD8\(^+\) T cells identified a proportion Csk at the proximal pole of the cell, colocalizing with p-Tyr, Lck, and Lck\(^{Y505}\) and Fyn (Fig. 4B). In addition, a large peak of both Csk and Fyn molecules was detected in close association with each other at the distal end of Ag-experienced (Fig. 4B), but not naïve (Fig. 4A), CD8\(^+\) T cells. Moreover, in Ag-experienced CD8\(^+\) T cells, the peak representing Csk at the distal pole was much
**FIGURE 2.** Naive and Ag-experienced CD8+ T cells differentially localize Csk and Fyn. Confocal immunofluorescence of resting naive (A) and Ag-experienced (B) CD8+ T cells stained with anti-TCRβ plus CD8α, DAPI, pY, Lck, LckpY505, and Fyn, as indicated. A single two-dimensional optical section (along x-y-axis) is shown in each panel with colocalization shown as a merge image of green and red pixels (fifth column). Scale bars, 3 μM (naive), 3.5 μM (Ag experienced). (C) Tables show values for colocalization of white (second column) and green (third column) pixels. Pearson correlation coefficient (Rr) was calculated using Velocity software from two independent experiments.

**FIGURE 3.** Csk and Fyn display bipolar distribution in Ag-experienced CD8+ T cells after TCRβ/CD8α-mediated activation. Confocal immunofluorescence of (A) naive and (B) Ag-experienced CD8+ T cells stimulated with TCRβ/CD8α for 5 min. Cells were fixed, permeabilized, and stained for Csk, pY, Lck, LckpY505, and Fyn, as indicated. A single two-dimensional optical section (along x-y-axis) is shown in each panel, and all red and green colocalized pixels are represented as white pixels on a merge image of 3+4. Scale bar, 3 μM (naive cells), 3.5 μM (Ag-experienced cells). (C) Tables show values for colocalization of white (second column) and green (third column) pixels. Pearson correlation coefficient (Rr) was calculated using Velocity software from at least two independent experiments (Rr 3+4 naive, n = 40; Rr 2+3 naive, n = 50; Rr 3+4, n = 50; Rr 2+3, n = 50).
In addition, we calculated the proportion of each molecule found in the proximal and distal poles of a cell by sectioning the merge image into the proximal end (P), identified by the TCRβ/CD8α cap, and the opposing distal end of the cell (D) (Fig. 4C). The results in Fig. 4C show clearly that Csk was significantly less polarized to the site of the TCRβ/CD8α cap in Ag-experienced CD8+ T cells than naive T cells (p ≤ 0.0001). Similarly, the majority of Fyn molecules were significantly more concentrated in the distal pole of stimulated Ag-experienced CD8+ T cells (Fig. 4C) compared with naive T cells (p ≤ 0.0001), further confirming the close association between Fyn and Csk. In addition, significantly more p-Tyr and Lck molecules were recruited to the TCRβ/CD8α cap in Ag-experienced rather than naive CD8+ T cells (p ≤ 0.01). These data show that distribution of signaling molecules changes between naive and Ag-experienced CD8 T cells such that the latter have tighter association of Lck, a positive regulator of T cell signaling, and exclusion of Csk, a negative regulator of signaling from the IS.

*Csk is sequestered from Lck and the TCR after activation*

To follow the kinetics of redistribution of proximal PTKs upon T cell stimulation of Ag-experienced CD8+ T cells, we used a high-throughput imaging flow cytometer, ImageStream, which although lacking the high resolution afforded by the confocal studies, enabled us to obtain a measure of the spatial distribution of molecules from large numbers of cells over multiple time points. The ImageStream allows the separation of cells based on multiple parameters including removal of apoptotic cells based on LIVE/DEAD staining so that only viable activated Ag-experienced CD8+ T cells were analyzed (gating strategy is detailed in Supplementary Fig. 1). A log-transformed Pearson correlation coefficient was used to measure colocalization (BDS score), and the morphometrically relevant biological control (37) of Lck and supplementary Fig. 1). A log-transformed Pearson correlation coefficient was used to measure colocalization (BDS score), and the morphometrically relevant biological control (37) of Lck and TCRβ/CD8α signals was used to set a threshold on two signals known to heavily colocalize. On this basis, we determined that median BDS scores < 1.5 (indicated by the broken line in the histograms, Fig. 5B) corresponded to dissimilar image pairs; conversely, scores > 1.5 were similar image pairs.

Analysis of Ag-experienced CD8+ T cells showed that at time 0, the localization of Csk significantly correlated with TCRβ/CD8α, Lck, and LckpY505 (BDS score ≥ 1.5; Fig. 5A, 5B), suggesting all proteins shared similar spatial location, in line with the confocal data (Fig. 2). After TCRβ/CD8α cross-linking, Lck retained a strong colocalization with TCRβ/CD8α (BDS score > 2), whereas 5 min after stimulation, the correlation between Csk with TCRβ/CD8α, Lck, and LckpY505 was lost (BDS score < 1.5). The only molecule with which Csk remained colocalized was Fyn (BDS score > 1.5) up to 40 min after activation. The loss of correlation between Csk and TCRβ/CD8α or Lck 5 min after TCR cross-linking (Fig. 5A) was due to the bipolar distribution of Csk in Ag-experienced CD8+ T cells as seen in confocal analysis (Fig. 3B). Interestingly, although LckpY505 maintained a strong colocalization with TCRβ/CD8α early after cross-linking beyond 20 min, this was reduced, so that by 40 min after TCR stimulation, the BDS score was ≤1.5, suggesting the negatively regulated form of Lck disappears from the TCRβ/CD8α cap over time. Overall, these data indicate that exclusion of Csk and Fyn from the site of TCR engagement is a dynamic process that is maintained for at least 40 min after activation.

*Csk localization is not dependent on Fyn*

Given the close association between Csk and Fyn in these studies, we asked whether the observed distribution of Csk required the presence of Fyn. Csk is recruited to the PM by interaction with

![Image](http://www.jimmunol.org/)
membrane-associated adaptor molecules including the transmembrane adaptor PAG (20, 21). In resting T cells, PAG is phosphorylated on Y314 by Fyn, generating an SH2 binding site for Csk (20). Previously, we showed that FynKO CD8+ T cells were more sensitive than wild-type (WT) cells to Ag stimulation, which correlated with abrogation of phosphorylation of PAGpY314 and was consistent with a loss of negative feedback (22). Therefore, we asked whether the spatiotemporal localization of Csk would be altered in Ag-experienced FynKO F5 CD8+ T cells. Somewhat surprisingly, there appeared to be no difference to WT cells in the localization of Csk in either unstimulated Ag-experienced FynKO F5 CD8+ T cells. We first asked whether its presence was influenced by the action of PTPs. Many intracellular PTPs act as negative regulators of TCR signaling, so we inhibited PTP activity with pervanadate (Na3VO4), which gives increased p-Tyr generating SH2-binding domains, which might alter Csk localization. Inhibition of cellular PTP activity by treatment of cells with Na3VO4 resulted in redistribution of Csk from the cytosol (indicated by arrows in unstimulated, Fig. 6C) to the cell periphery, with almost complete loss of the cytosolic structure. These data argue that compartmentalization and sequestration of Csk from the PM is maintained by PTPs playing a bipolar distribution (Fig. 6A). Quantification of the distribution of Csk molecules in polarized WT and FynKO Ag-experienced CD8+ T cells showed no significant difference between the two genotypes (Fig. 6B). These data suggest that the bipolar distribution of Csk observed in Ag-experienced CD8+ T cells is not dependent on PAGpY314.

To investigate the localization of Csk at the distal structure, we first asked whether its presence was influenced by the action of PTPs. Many intracellular PTPs act as negative regulators of TCR signaling, so we inhibited PTP activity with pervanadate (Na3VO4), which gives increased p-Tyr generating SH2-binding domains, which might alter Csk localization. Inhibition of cellular PTP activity by treatment of cells with Na3VO4 resulted in redistribution of Csk from the cytosol (indicated by arrows in unstimulated, Fig. 6C) to the cell periphery, with almost complete loss of the cytosolic structure. These data argue that compartmentalization and sequestration of Csk from the PM is maintained by PTPs.
activity in Ag-experienced CD8⁺ T cells. Staining with γ-tubulin showed that the centrosome was present in the distal area of the Ag-experienced cell in which Csk was found to accumulate (Fig. 6D). Somewhat surprisingly, the centrosome did not reallocate to the cap after TCR plus CD8 cross-linking (Fig. 6D). It has been shown that the centrosome localizes to the IS after Ag stimulation of CTL, and that docking of the centrosome at the IS requires signals from Lck (38). We confirmed that the formation of Ag-specific conjugates between Ag-experienced CD8 T cells and peptide-pulsed RMA-S cells relocated the centrosome to the IS (Fig. 6D). In addition, we followed the location of Csk in Ag-specific conjugates and found that Csk was distributed at both the IS and, to a lesser extent, the distal pole of the cell at the 5-min time point (Fig. 6D). By 10 min of conjugation, most Csk molecules were associated with the IS and this association became more localized over time (Supplemental Fig. 3B). Therefore, the redistribution of Csk and the centrosome is influenced by the nature of the stimulus, and surprisingly, despite the observation that cross-linking with TCR and anti-CD8 Abs causes robust Lck phosphorylation, this is insufficient to relocalize the centrosome to the site of capping.

These results identify differences in the distribution of signaling molecules in naive and Ag-experienced CD8⁺ T cells both in the resting state and after TCR activation with Ab stimulation. Taken together, they indicate that loss of the critical negative regulator Csk from the PM reduces the threshold of TCR triggering in effector/memory CD8⁺ T cells. Furthermore, upon TCR triggering with Abs, Csk and the centrosome remain sequestered at the distal pole of the cell.

**Discussion**

The molecular basis for the increased sensitivity of memory T cells to TCR triggering by Ag is currently unknown. Using confocal imaging of endogenous molecules, we addressed this issue by asking whether we could see differences in the distribution of key proximal signaling molecules between naive and Ag-experienced CD8⁺ T cells, in both the resting state and after TCR engagement. We show that Csk, the primary negative regulator of the SFKs, Lck and Fyn, has a unique cytoplasmic location in Ag-experienced cells, which was not seen in naive CD8⁺ T cells. In addition, there was a pool of Csk associated with the

![FIGURE 6](http://www.jimmunol.org/)

(A) Csk localization does not require PAGpY³¹⁴. (B) The sum of fluorescence above background of each condition was calculated using Velocity software in both the proximal and the distal half of the cell. The data set of 1 experiment, comprising 25 images for each condition, was used to generate the protein distribution graph and calculate p values as determined by the Student two-tailed t test which were not significantly different. Data are representative of two independent experiments. (C) Ag-experienced CD8⁺ T cells were treated for 20 min with Na₃VO₄, stained with TCRβ/CD8α, fixed, permeabilized, and stained for Csk, with nuclei stained with DAPI. A single two-dimensional optical section (along x–y-axis) is shown in each panel, and an overlay of red and white pixels is represented as a merge image. Scale bar, 1.3 μM. Arrows indicate distribution of Csk in cytoplasm. Data are representative of at least 50 cells of each condition from three independent experiments. (D) Ag-experienced CD8⁺ T cells were conjugated to NP68- or GAG-pulsed RMA-S cells for 5 min (top two panels) or activated by TCRβ/CD8α cross-linking (bottom two panels) and fluorescently labeled with Abs to Csk and γ-tubulin to label the centrosome. Images were rendered with Velocity software. Representative conjugates containing GAG-pulsed RMA-S (first panel) or NP68-pulsed RMA-S (second panel) each intracellularly labeled with MitoTracker (white) and a single Ag-experienced CD8⁺ T cell. Scale bar, 6 μM. Data are representative of at least 50 cells of each condition from two independent experiments.
cell periphery, which was common to both cell types. Upon TCR engagement, the discrete cytoplasmic pool of Csk was maintained in the distal pole of the cell away from the TCR. Fyn, but not Lck, was associated with the cytoplasmic pool of Csk, whereas both Fyn and Lck were associated with peripherally distributed Csk. However, there was no absolute requirement for Fyn to maintain the cytoplasmic pool, as it was undiminished in FynKO Ag-experienced CD8+ T cells. Our data support the hypothesis that the reduced threshold of activation seen in Ag-experienced CD8+ T cells could be facilitated by the spatial redistribution of proximal signaling mediators, such as sequestration of Csk into the cytosol away from its substrate Lck at the PM.

We showed previously that the availability of Lck was an important element in determining the activation threshold of primary T cells (36). More recently, it was suggested that the activity of Lck was crucial for initiating T cell activation (30). Up to 40% of Lck in resting T cells was found to be constitutively active, and a proportion of this activated pool was phosphorylated on the regulatory Y505 residue, previously thought to mark only inactive pools of Lck (30). Csk is the PTK responsible for phosphorylating the regulatory Tyr but, unlike the SFKs, Csk is not constitutively targeted to the PM. Instead, Csk is recruited by interaction of its SH2 domain with a variety of Tyr phosphorylated proteins (15, 39–43). Constitutive targeting of Csk to the PM reduced the basal state of activity of primary T cells (16), indicating that the localization of Csk is important in regulating the signaling threshold even in the absence of Ag.

The presence of a distal pole complex (DPC) that forms early after TCR activation has been previously identified in CD4+ T cells (44–46), and was shown to concentrate negative regulators of T cell signaling, including SHP-1 and PAG. Localization of PAG to the DPC was potentially mediated by Ezrin, as EBP-50, which is the intermediary protein linking PAG to the actin cytoskeleton through Ezrin, was also localized to the DPC (47). However, the arrangement of molecules in the DPC was different from that observed in this study, because the DPC was distributed as if attached to the PM, whereas we found Csk to have a distinct cytoplasmic location. In addition, molecules were reported to localize to the DPC only after pMHC1 Ag presentation (44, 45, 47), whereas we found Csk colocalizing in the cytoplasmic pool in Ag-experienced CD8+ T cells to be constitutive and unchanged by Ab-mediated TCR cross-linking. Upon interaction with APC and formation of conjugates, T cells round up as they receive stop signals (48, 49). The assembly of the IS occurs in various stages, which depend on distinct cytoskeletal rearrangements that facilitate the integration of signals, but the requirement of the IS may vary between CD4+ and CD8+ T cells, and whether the cells are naive, have effector functions, or are multifunctional memory cells. In naive CD4+ T cells, microclusters containing CD3, TCR, and PLCγ aggregate within the Supra Molecular Activation Cluster, the site of T cell signaling. In CTL, a correlation between the formation of the distal Supra Molecular Activation Cluster and the docking of the centrosome at the PM has been demonstrated, the latter required for granule delivery and lytic function (50). The signals delivered through TCRβ/CD8α cross-linking were not sufficient to polarize nonmigratory Ag-experienced CD8+ T cells, perhaps reducing their full effector function or limiting their multifunctional potential. Regardless, early signaling events immediately downstream of the TCR were enhanced in Ag-experienced CD8+ T cells compared with naive T cells, which may be attributed to the removal of Csk from the PM and its active sequestration within a cytoplasmic structure both in a basal state and after TCR triggering to dynamically regulate Csk activity, possibly by inhibiting activation by PKA at the PM (51).

It was striking that Fyn showed a very tight colocalization with Csk both at the cell periphery and in the cytoplasmic pool, unlike Lck, which was only colocalized with Csk at the PM. Fyn is responsible for phosphorylating PAG314, which is the major p-Tyr residue that recruits Csk (20, 52). We were unable to determine whether PAG colocalized with Csk in the cytoplasmic pool, because available anti-PAG Abs gave excessive background staining in confocal microscopy. Certainly Fyn-mediated PAG phosphorylation was not essential for this localization of Csk, because FynKO Ag-experienced cells showed similar distribution of Csk to WT CD8+ T cells. However, the absence of an overtly deleterious phenotype in PAGKO mice (53, 54) and in FynKO mice (55, 56) argues that molecules other than Fyn and PAG are also able to influence Csk localization.

In this study, we addressed the requirement of key signaling mediators associated with TCR-dependent activation and therefore triggered the TCR through Ab cross-linking of CD3, or TCR plus CD8 in the absence of costimulation, which has been reported to affect IS formation between naive and memory T cells (57). It has been shown previously that Lck associates with the coreceptor (3, 58), whereas Fyn interacts with TCR/CD3 (59). Indeed, FynKO T cells became hyporesponsive upon anti-CD3 stimulation (55, 56), most likely because Lck was not activated to the same extent as when CD3 was engaged in combination with anti-CD4/CD8 (60). In support, our data identified that anti-CD3 alone was largely inadequate at redistributing Lck from around the cell periphery to the CD3 cap, compared with TCR, which generated a 3-fold increase in Lck coincident with the TCRβ cap. It was not until additional CD8 colligation that maximal colocalization of Lck was observed in both naive and Ag-experienced CD8+ T cells. Of interest, Ag-experienced CD8+ T cells were twice as efficient in redistributing Lck as naive CD8+ T cells, which is consistent with previous findings that a greater proportion of Lck molecules are found at the PM associated with the coreceptor in memory CD8+ T cells (61). This suggests that there is a rapid reorganization of positive mediators of TCR signaling in Ag-experienced compared with naive CD8+ T cells, which may contribute to the enhanced memory cell response.

Our data are consistent with the requirement for specific compartmentalization of signaling mediators for the regulation of T cell homeostasis and the altered sensitivity to Ag stimulation that occurs between naive and Ag-experienced CD8+ T cells. We propose that early TCR triggering events drive divergent signaling events between naive and memory CD8+ T cells, and it is the rapid reorganization of signaling molecules that contributes to enhanced memory cell responses.

Acknowledgments
We thank Derek Davies (London, U.K.) for discussion of ImageStream flow cytometry analysis and members of the Zamoyska laboratory (Edinburgh, U.K.) for general discussions.

Disclosures
The authors have no financial conflicts of interest.

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