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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: 000–000.
importance of cellular localization for Csk function. Furthermore, loss of PACGR334 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 pMHC 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, PACGR334 and Csk colocalized at the PM, which correlated with increased phosphorylation of 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 PTKs 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 LckY505 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, 1-glutamine, 100 U/ml penicillin and streptomycin (Life Technologies), and 50 μM 2-ME, additionally supplemented with 10 nm NP68 for 3 d. The NP68 nonamer corresponds to residues 366–374 of nucleoprotein from strain A/NT schematica/68 and is the naturally occurring epitope presented to T cells by H-2Db (27). Activated CD8+ T cells were then rested for 4 d in 5 ng/ml IL-2 and 10 ng/ml IL-15 (all from PeproTech, London, U.K.).

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 37°C for T 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 × 105 cells/well in complete media containing soluble peptide at concentrations of 10−6 to 10−11 M NP68 or 10−7 M control peptide from the GAG protein of the SF2 strain of HIV (aa 390–398). Alternatively, 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 streptavidin 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.

T cell–APC conjugation assay

RMA-S cells were incubated overnight at 1 × 106/ml in RPMI/10% FCS in a 25°C water bath to enable stable expression of the MHC I molecules at the cell surface. Cells were pulsed for 30 min with 1 μM NP68 or GAG at 25°C, then returned to 37°C/5% CO2 for a minimum of 3 h to facilitate the decay of empty MHC I molecules from the surface. CD8+ T cells were preincubated on coverslips for 15 min at 37°C/5% CO2. Pulsed RMA-S cells were labeled with 1 μM CellTracker CMTPXR (Molecular Probes), at 5 × 107/ml, for a minimum of 30 min at 37°C/5% CO2. Labeled cells were washed twice in complete media and incubated for 3 h at 37°C/5% CO2 to eliminate excess dye. RMA-S cells were mixed with T cells and centrifuged for 1 min at 800 rpm to commence conjugate formation. Cells were then plated at 37°C/5% CO2 for the indicated time points, until fixed by addition of 4% PFA.

Flow cytometry

Cells were washed twice with 1× PBS, then incubated in LIVE/DEAD (Invitrogen) for 10 min at room temperature (RT). Cells were then incubated with Para-Fix blocking mix [24G2, BioLegend] for 10 min at RT and then stained 30 min at 4°C with PE-labeled anti-Vb11 (eBiosciences). For detection of intracellular Tyr phosphorylated proteins, cells were fixed in 2% final concentration PFA for 30 min on ice, washed and permeabilized in 90% ice-cold methanol for 30 min on ice, then stained with anti–phospho-p44/42 MAPK (Erk1/2), T202/Y204; clone 197G2; Cell Signaling) for 1 h at RT. Cells were washed twice in FACS buffer washed and incubated with a second-layer streptavidin conjugated to an AF (Molecular Probes) for 30 min at RT, washed and resuspended in FACS buffer for acquisition on FACS/CALibur or LSR II cytometers (BD). Data were analyzed in FlowJo software (Tree Star, Ashland, OR).

Confocal microscopy

Cells were stimulated as described earlier and fixed in 4% paraformaldehyde, with residual PFA quenched in 50 mM glycine for 10 min at RT. Cells were permeabilized in 0.1% Triton X-100 for 6 min at RT, washed 4 times in 1× PBS, before staining with anti-Lck (Cell Signaling), anti–phospho-Lck Y505 (Cell Signaling), anti–Csk (Abcam) and anti-Fyn (Millipore), anti–γ-tubulin (Sigma), and BODIPYFL phallacidin (Invitrogen) at 4°C overnight in PBS/0.5% BSA. Cells were washed four times in PBS/0.2% BSA and further incubated with species-specific anti–Fab(′)2-AF488 or 647 conjugates (Molecular Probes) for 15 min at 4°C. The secondary Ab was removed by washing four times in PBS/0.2% BSA, followed by a 1× PBS wash. Nuclei were stained with DAPI supplemented at 1 μg/ml in ProLong Fade Gold (Invitrogen) mounting media. Samples were examined on the Leica SP1 II (Leica Microsystems) with lasers exciting at 405, 488, 543, and 647 nm with the 63x objective, using LAS AP software (Leica). Representative images of the localization of each molecule are shown. All confocal analyses were multiple repeats, and at least 50 images were analyzed for each molecule. Data were rendered and analyzed using Velocity software (Improvision) and ImageJ (National Institutes of Health). Pearson correlation coefficient was calculated using Velocity software to determine the pairwise colocalization of the signals with a Student paired two-tailed t test used to determine statistical significance between two protein data sets.

For quantitation of protein distribution within a single-cell image, a line parallel to the plane of the TCR cap was used to identify a 50% region of the cell, termed as proximal, with the distal half of the cell posterior to the site of TCR capping termed distal. A background threshold was set for the channel to be measured and then applied to each image, and the sum of fluorescence in each region was determined using Velocity software. The sum of fluorescence above the threshold minimum generated by the imaging software was recorded and entered into the following formula: (P − D) / (P + D), where P is the fluorescence in the proximal half of the cell and D is the fluorescence in the distal half of the cell. A Student paired two-tailed t test was used to determine statistical significance between two protein data sets.

ImageStream analysis

Samples were acquired on a five-laser, six-channel ImageStream X (Amnis, Seattle, WA) imaging flow cytometer with violet (120 mW), blue (100 mW),
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, SKF 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–efficient–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-2Dβ (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 CD3ε 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 CD3ε alone, TCRβ alone, or TCRβ plus CD8α drove discrete capping in both naive and Ag-experienced CD8+ T cells as expected (Fig. 1A). In naive CD8+ T cells, cross-linking CD3ε alone caused only a small proportion of cells (6%) to redistribute Lck to the CD3ε 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-capping 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 CD3ε 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-colligation, 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 negative 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). Naive 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 engagement (Fig. 2A). 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, 0.75 and 0.78, respectively; Fig. 2A). Because Csk is the only kinase to phosphorylate Lck\(^{Y505}\) (7, 24), the relative distribution of Csk and LckpY505 was investigated. In naive CD8\(^+\) T cells, LckpY505 was homogenously distributed at the cell periphery, highly colocalized with Csk (Rr = 0.81; Fig. 2A).

In Ag-experienced CD8\(^+\) T cells, basel levels of p-Tyr were again detected at the cell periphery together with Lck (Fig. 2B). Strikingly, in contrast with naive cells, Csk distributed not only with SFKs at the cell periphery of Ag-experienced CD8\(^+\) T cells, but was also observed within a discrete cytoplasmic area in the majority of cells analyzed (Fig. 2B). As a consequence, the average localization score between Csk with Lck and Csk with LckpY505 decreased significantly in Ag-experienced compared with naive CD8\(^+\) T cells (p ≤ 0.05 and p ≤ 0.01, respectively). Interestingly, the correlation between Csk and Fyn remained similar between naive and Ag-experienced CD8\(^+\) T cells (Rr = 0.78 and 0.77, respectively; p > 0.05; Fig. 2A, 2B). In Ag-experienced CD8\(^+\) T cells, Fyn distributed similarly to Csk, both at the cell periphery and within the cytosolic area in which Csk was present (Fig. 2B). Similarly to naive cells, Csk demonstrated a significant loss of association with TCRβ/CD8\(\alpha\) (Rr = 0.74, p ≤ 0.0001; Fig. 2C) and maintained stronger colocalization with Fyn compared with PM-associated Lck (Rr = 0.77 and 0.62, respectively), reflecting the increased accumulation of Csk with Fyn within the defined cytosolic area. We asked whether the differential localization of Csk in Ag-experienced versus naive CD8\(^+\) T cells resulted in altered Lck activity, by examining phosphorylation of Shc, which is a target of Lck (13). Ag-experienced CD8\(^+\) T cells exhibited faster kinetics and greater phospho-Shc after stimulation, consistent with stronger activation of Lck in this population compared with naive CD8 T cells (Supplemental Fig. 1).

### Bipolar distribution of Csk and Fyn upon TCR engagement of Ag-experienced, but not naive 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 naive CD8\(^+\) T cells, we asked how Csk and Fyn behaved upon TCR stimulation in both cell types. After TCRβ/CD8\(\alpha\) cross-linking, naive T cells showed strong association of Csk with p-Tyr, Lck, LckpY505, 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 LckpY505 (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 TCRβ/CD8\(\alpha\) cap even after TCR cross-linking. As a consequence, although the capped TCR remained as, or even more, tightly associated with p-Tyr, Lck, and LckpY505, the association between TCRβ/CD8\(\alpha\) and Csk reduced from Rr = 0.51 in naive 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 naive 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 naive 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 naive CD8\(^+\) T cells, the majority of Lck, LckpY505, Fyn, and Csk molecules polarized to the site of the TCRβ/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 LckpY505 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 naive (Fig. 4A), CD8\(^+\) T cells. Moreover, in Ag-experienced CD8\(^+\) T cells, the peak representing Csk at the distal pole was much smaller.

### 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>
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</thead>
<tbody>
<tr>
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<td>Naive</td>
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<td>27</td>
<td>6.1</td>
</tr>
<tr>
<td></td>
<td>pY</td>
<td>Ag experienced</td>
<td>1385</td>
<td>16</td>
<td>13</td>
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<td>1637</td>
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<tr>
<td></td>
<td>pY</td>
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<td>760</td>
<td>34</td>
<td>40</td>
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<tr>
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<td>1325</td>
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</tr>
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<td></td>
<td>pY</td>
<td>Ag experienced</td>
<td>826</td>
<td>23</td>
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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.
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, Csk, pY, Lck, Lck<sup>Y505</sup>, 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 (R<sub>r</sub>) 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, Lck, Lck<sup>Y505</sup>, and Fyn (bottom row), Lck<sup>Y505</sup> (third row), and Fyn (bottom row). 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 (R<sub>r</sub>) was calculated using Velocity software from at least two independent experiments (R<sub>r</sub> 3+4 naive, n = 40; R<sub>r</sub> 2+3 naive, n = 50; R<sub>r</sub> 3+4, n = 50; R<sub>r</sub> 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 Fyn was lost (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...
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 CD8+ T cells or after TCRβ/CD8α-mediated activation (Fig. 6). In both WT and FynKO Ag-experienced CD8+ T cells, Csk was identified at the periphery of the cell, as well as localizing within a distinct cytosolic pool (Fig. 6A). After TCRβ/CD8α cross-linking, there was no difference in the localization of Csk between WT and FynKO T cells, with Csk displaying 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 PTP

**FIGURE 5.** Csk is maintained away from the site of TCR activation in Ag-experienced CD8+ T cells. Ag-experienced CD8+ T cells were stimulated with TCRβ/CD8α and streptavidin AF405 (column 1) for the indicated times (0–40 min), fixed, permeabilized, and intracellular proteins were stained for Csk, column 3, and in column 1 for Lck (A), LckY505 (B), or Fyn (C). Each panel shows representative cells from the median BDS-R3 value at 0 and 5 min, with colocalization shown as a merge image of green and red pixels (2 + 3). Graphs of the median BDS-R3 score for the indicated image pairs on a per cell basis are the mean of three independent experiments ± SEM (for some points, the error bars are too small to be visible without the symbols). The dotted line denotes the morphometrically relevant biological–determined threshold below which two signals are considered to be dissimilar by the BDS-R3 score (<1.5).
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 relocalize 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

fixed, permeabilized, and intracellular proteins were double stained for Csk with Lck and LckpY505. (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 3 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.
signals delivered through TCR.

The latter required for granule delivery and lytic function (50). The docking of the centrosome at the PM has been demonstrated, the formation of the distal Supra Molecular Activation Cluster and Cluster, the site of T cell signaling. In CTL, a correlation between the integration of signals, but the requirement of the IS may state the integration of signals, but the requirement of the IS 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 acting 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 PAGY314, 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 in memory CD8+ T cells (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 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 associated with TCR-dependent activation and there-fore 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 in memory CD8+ T cells (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 associated with TCR-dependent activation and there-


Supplementary Figure 1: Enhanced responses of Ag.experienced CD8\(^+\) T cells upon TCR stimulation

LN cells from Rag-1\(^{-/-}\)F5 mice (naïve) and Ag.experienced CD8\(^+\) T cells generated by stimulation with 10\(^{-7}\)M NP68 for 72 h then rested in IL-2 and IL-15 for 96 h, were activated with peptide (NP68) and their responses were assessed. (A) The percentage of CD8\(^+\)cells that downregulate Vβ11 TCR in response to a titration of peptide at 3 h is shown. Histogram shows expression of surface Vβ11 TCR at 10\(^{-9}\)M NP68 in naïve unstimulated T cells (filled); Ag-experienced unstimulated level (dashed line); naïve peptide stimulated (blue); and Ag-experienced peptide stimulated (red). Data are representative of at least 6 mice of each phenotype from two independent experiments. Values are the mean of 6 data points ± SD. (B) Histograms show the levels of pERK following 5 and 20 min 10\(^{-9}\)M NP68 or TCR/CD8 crosslinking (Ab X-link) in naïve unstimulated T cells (filled); Ag.experienced unstimulated (dashed line); naïve peptide stimulated (blue); and Ag-experienced peptide stimulated (red). Data are representative of at least 6 mice of each phenotype from 2 independent experiments. Values are the mean of 6 data points ± SD. (C) T cells were stimulated for indicated times with 10\(^{-7}\)M NP68 and whole cell lysates run on Tris-Bis gels and immunoblotted for pSHC Y239/40 and total SHC indicates loading. Relative pSHC levels were determined by normalizing data of phospho-SHC to total SHC, with densitometry quantitation by infrared imaging using the LI-COR Odyssey system.
Supplementary Figure 2: Imaging Flow Cytometry gating and analysis strategy

Gating and analysis strategy for measuring pair-wise co-localisation of TCR/CD8 (AF450), SFK (AF488) and Csk (AF647) signals on live, single, in focus, triple stained cells. (A) Single, live cells were defined using the area (x-axis) and aspect ratio (y-axis) of the bright field (BF) channel default mask (M04). Single cells have a high aspect ratio and intermediate area whereas doublets have lower aspect ratio and double area values (see example images). (B) Poorly focused events were eliminated with “gradient RMS” calculated from the pixels identified by the M04 mask. Values above 200 units were considered to be in focus. Representative images are shown of events within and without the focused gate. (C) TCR/CD8-AF450 and SFK-AF488 double positive cells were gated using the “total intensity” feature which sums the pixel values in a given channel mask AF450 (M01), x axis and AF488 (M02), y axis. Values are plotted on a log scale. (D) Identification of triple positive cells using the sum pixel value of the AF647 (M05) signal on a log scale (y-axis). (E) The BDS score was calculated using the complete mask (MC) and the AF450 versus AF488 channel pixel values (X-axis), the AF488 versus AF647 channel pixel values (y-axis) or the (F) AF488 versus AF647 channel pixel values (y-axis). In all cases, median values were reported for the triple positive populations. A dissimilar cut off value of <1.5 units was determined using the MRB control of TCR/CD8-AF450 versus SFK-AF488 signals as a highly correlated distribution. The BDS score functions by first subtracting away all light from sub-optimal focal planes using a 3 pixel-structuring element (R3) so that only bright-detail pixels within the plane of best focus are used for Pearson’s correlation. This minimises co-localisation on the x and y-axis of a 2D slice being confounded by a lack of z resolution. In all cases gated percentages (%) are shown and median values (SEM) included for BDS scores. Compensation values generated from single stained controls post-acquisition for Ch1, 2, 3, 4, 5 and 6 respectively were AF450:100, 16.6, 4.1, 2.7, 1.1, 0.6; AF488 11.3, 100, 20, 7.5, 1, 0.25; AF647: 6, 8, 6, 1, 100, 20.
Supplementary Figure 3: Immunological synapse formation is required for Csk redistribution in Ag.experienced CD8\(^+\) T cells
Ag.experienced CD8\(^+\) T cells were conjugated to NP68- or GAG-pulsed RMA-S cells for 5-40 min, and conjugates were fixed and fluorescently labeled with Abs to Csk and BODIPYFL-phalloidin to label F-actin accumulation at the SMAC. (A) Representative conjugates containing GAG-pulsed RMA-S (top panel) or NP68-pulsed RMA-S (bottom panel) each intracellularly labelled with Mitotraker (white) and a single Ag.experienced CD8\(^+\) T cell. Scale bar represents 6 µM. (B) Using Volocity software, the sum of fluorescence above background of each time point was calculated in both the proximal and distal half of the cell. The amount of Csk fluorescent units were entered into the formulae \((P-D)/(P+D)\). The data set of 1 experiment, comprising 25 images for each condition. The data are representative of at least 2 independent experiments.