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Constitutive Lck Activity Drives Sensitivity Differences between CD8+ Memory T Cell Subsets

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CD8+ T cells develop increased sensitivity following Ag experience, and differences in sensitivity exist between T cell memory subsets. How differential TCR signaling between memory subsets contributes to sensitivity differences is unclear. We show in mouse effector memory T cells (TEM) that >50% of lymphocyte-specific protein tyrosine kinase (Lck) exists in a constitutively active conformation, compared with <20% in central memory T cells (TCM). Immediately proximal to Lck signaling, we observed enhanced Zap-70 phosphorylation in TEM following TCR ligation compared with TCM. Furthermore, we observed superior cytotoxic effector function in TEM compared with TCM, and we provide evidence that this results from a lower probability of TCM reaching threshold signaling owing to the decreased magnitude of TCR-proximal signaling. We provide evidence that the differences in Lck constitutive activity between CD8+ TCM and TEM are due to differential regulation by SH2 domain–containing phosphatase-1 (Shp-1) and C-terminal Src kinase, and we use modeling of early TCR signaling to reveal the significance of these differences. We show that inhibition of Shp-1 results in increased constitutive Lck activity in TCM to levels similar to TEM, as well as increased cytotoxic effector function in TCM. Collectively, this work demonstrates a role for constitutive Lck activity in controlling Ag sensitivity, and it suggests that differential activities of TCR-proximal signaling components may contribute to establishing the divergent effector properties of TCM and TEM. This work also identifies Shp-1 as a potential target to improve the cytotoxic effector functions of TCM for adoptive cell therapy applications. The Journal of Immunology, 2016, 197: 000–000.

T cell effector functions are initiated by ligation of the TCR with an MHC-presenting Ag peptide (pMHC) on the surface of an APC (1). T cell sensitivity is substantially increased following Ag experience, and maturation and can vary between Ag-experienced memory subsets (2), which has been attributed in part to enhanced TCR-proximal signaling (3). Central memory (TCM) and effector memory (TEM) T cells have unique gene expression and cytokine signaling signatures (4), which result in distinct effector capacities (5). As a result, TCM have an enhanced ability to confer host protection against viral and bacterial challenge (6) as well as enhanced therapeutic antitumor responses compared with TEM (7). However, TEM possess greater in vitro cytotoxic properties (8), which suggests that the superior in vivo properties of TCM result from greater proliferation upon Ag re-encounter and preferential homing to secondary lymphoid tissues (7, 9) despite a deficiency in cytotoxic properties compared with TEM (9). The contributions of TCR signaling components that confer differences in activation sensitivity and functional outcomes between CD8+ TCM and TEM remain unclear.

Initiation of T cell signaling by TCR ligation leads to a sequence of well-characterized signaling events, including lymphocyte-specific protein tyrosine kinase (Lck) phosphorylation of CD3 ITAMs (10) and Zap-70 (11). Active Lck is present in T cells prior to TCR stimulation (12) and exists in equilibrium between four states, based on phosphorylation of activating Y394 and inhibitory Y505 (12). It is unclear whether the level of constitutively active Lck differs significantly between T cell subsets, and whether any such differences in Lck activity would contribute to establishing...
differential Ag sensitivities. This premise is supported by recent work by Manz et al. (13), which showed that increasing Lck activity through inhibition of C-terminal Src kinase (Csk) leads to enhanced downstream signaling following T cell stimulation. In the present study, we show that TCM and TEM possess differential constitutive Lck activities, driven in part by differential regulation by SH2 domain–containing phosphatase-1 (Shp-1) and Csk. In response to the moderate affinity (9.3 μM) self-antigen gp100209–217, differences in proximal T cell signaling resulted in significantly different probabilities of TCM and TEM achieving full cytotoxic effector function. Comparatively higher constitutive Lck activity can explain the more robust proximal Ag-dependent signaling and cytotoxic effector function of TEM. Given the importance of both TCR dwell time and Lck in driving TCR signaling (14), our results suggest that T cell sensitivity may be influenced by constitutive Lck activity, which varies sufficiently between TCM and TEM to establish differential Ag sensitivities.

Materials and Methods
Reagents and materials
All cells were cultured in RPMI 1640 l-glutamine–supplemented media (Life Technologies) with the inclusion of 10% FBS (Thermo Scientific), sodium pyruvate, MEM nonessential amino acids, and penicillin/streptomycin (Life Technologies). Anti-Lck (SPM413 and 2102), anti-pY394-Lck (Tyr394), anti-pTyr656 Lck (pY505.4), anti-GST (K-18), anti-Zap-70 (E7.2), anti–Shp-1 (C19), anti-Csk (C-20), anti–Csk-binding protein (Cbp; PAG-C1), anti–rabbit IgG F(ab')2–allophycocyanin, anti–mouse IgG F(ab')2–FITC, and normal rabbit IgG isotype control were from Santa Cruz Biotechnology. FITC-conjugated anti-CD8α (53-6.7), PerCy5.5–conjugated anti-CD44 (IM7), and PE-conjugated CD62L (MEL-14) were from eBioscience. Anti–pShp-1 (S591) and anti–pShp-1 (Y536) were from ECM Biosciences. Anti–pShp-1 (Y536) was from Life Technologies. Anti–pShp-1 (Y564) were from Cell Signaling Technology. Anti–pY142 (K25–407.69) and Anti–Them (Q1-1103) were from BD Pharmingen. Anti–rabbit IRDye 680LT, anti–goat IRDye 680LT, and anti–mouse 800CW IRDye were from LI-COR Biosciences. Allophycocyanin–anti–mouse IgG (poly-4053) was from BioLegend. Anti–phosphorosynsine (4G10) was from Millipore. Anti–actin was from Acris Antibodies. Anti–Chp (EPR9705) was from Abcam. CD3–Cdx-7 with N-terminal GST tag was from Novus Biologicals. ProLong Gold antifade with DAPI, funa 2-AM, Lysotracker Red, and calcine green AM was from Life Technologies. gp100209–217 (2M) peptide (IMDQVPFSV) was from Bio-Synthesis.

Animals and cell culture
JR209 TCM and TEM were obtained as previously described (15). Major lymph nodes and spleens were extracted from the humanized transgenic JR209 mouse (16) and mechanically digested, followed by lysis of the RBC population with ACK lysis buffer (Life Technologies). Isolated lymphocytes were cultured at an initial concentration of 5 × 10⁶ cells/ml with 1 μM gp100209–217 (2M) and 20 ng/ml IL-15 or IL-2 (R&D Systems) (17, 18) and passaged 1:2 every other day with replenishment of media and 10 ng/ml cytokine. At day 6 of culture, live cells were purified by density gradient centrifugation with Ficoll-Paque Plus (GE Healthcare), washed and resuspended at 2 × 10⁶ cells/ml, and allowed to rest for at least 3 h or overnight before use in serum-free media. The phenotypes of TCM (CD44hi, CD62Llo) and TEM (CD44hi, CD62Lhi) were verified by flow cytometry.

Chromium release cytotoxicity assay
Bulk T cell cytotoxicity was assessed by chromium release assay, as previously described (19). Peptide-loaded (at indicated concentrations), ⁵Cr-labeled T2-A2Kb APCs and T cells were cultured in round-bottom 96-well plates at the ratios indicated for 6 h at 37°C. Positive control (maximum release) wells included 5% Triton X-100 solution in the absence of T cells, whereas negative control (spontaneous release) wells contained APCs only. Supernatant (100 μl) from each well was transferred to a 96-well isoplate (PerkinElmer) and 100 μl OptiPhase SuperMix scintillation fluid (PerkinElmer) was added and thoroughly mixed and gamma radiation detected with a MicroBeta2 microplate counter (Perkin-Elmer). Specific lysis was calculated as (counts per minute of experimental release – counts per minute of spontaneous release)/(counts per minute of maximum release – counts per minute of spontaneous release). For quantification of cytotoxicity following Shp-1 inhibition, cells were treated with 50 μM NSC-87877 (Calbiochem) overnight and washed three times prior to addition of APCs.

Quantification of effector molecule expression by RT-PCR
Total RNA was extracted from resting T cells using a RNeasy kit (Qiagen). cDNA was synthesized using a first-strand cDNA synthesis kit (Roche) using oligo-oligo(dT)₅ primer. Quantitative PCR and analysis were performed using the LightCycler 480 (Roche) with Probes Master and TaqMan probes (Life Technologies) specific for granzyme B, perforin, and Fas ligand and hypoxanthine phosphoribosyltransferase as housekeeping gene. Relative expression was quantified as fold change in expression relative to naive T cells.

Granule polarization imaging
T cells were labeled with 50 nM Lysotracker Red in complete media for 30 min at 37°C. T cells and peptide-loaded APCs were combined in phenol red–free RPMI 1640 plus 10% FBS in a single well of an eight-chambered borosilicate coverglass system (Nunc), treated with 10% poly-l-lysine for 10 min (Sigma-Aldrich). Differential interference contrast (DIC) and fluorescence images were collected at 20-s intervals for 6 h with an Axiovert 200D inverted microscope (Zeiss) and ×40 oil objective (EC Plan-Neofluar, 0.6 numerical aperture, Zeiss) housed at 37°C/5% CO₂. Images were acquired with a CoolSNAP HQ camera (Photometrics). For analysis, T cell/ APC conjugates were orientated using DIC images and a grid was created to identify the quadrant of the T cell that included the T cell/APC interface. The polarization time was determined as the time when 80% of the Lysotracker signal is in the front quadrant. Image acquisition and analysis were performed using MetaMorph microscopy automation and image analysis software (Molecular Devices).

Time-lapse imaging microscopy in nanowell grids
For the time-lapse imaging microscopy in nanowell grids assay for detection of serial killing events, T cell/APC interactions and cytotoxic kinetics were performed on microfabricated nanowell, as previously described (20, 21). Nanowell arrays were fabricated on a glass-bottom petri dish (Ted Pella, 14027-200). T cells and peptide-loaded T2-A2Kb APCs were labeled with PKH67 and PKH26 dyes (Sigma-Aldrich), respectively. Furthermore, TEM was additionally labeled with Vybrant violet dye (Life Technologies) to differentiate between TCM and TEM. Subsequently, TCM, TEM, and APCs were loaded onto nanowell arrays and imaged for 6 h at 5-min intervals under 37°C/5% CO₂ with a ×20 air objective (Plan-Apochromat ×20/0.8 Ph2 M27) and ORCA flash 4.0 camera (Hamamatsu). Annexin V–Alexa Fluor 647 (Life Technologies) was added to dynamically track APC apoptosis. Raw images were analyzed using scripts for automated image preprocessing and segmentations (22). Resulting data were then analyzed using Excel, Access, and GraphPad Prism to identify and record serial killing events of interest.

 Imaging of T cell cytotoxic efficiency
For imaging of T cell cytotoxic efficiency, T cell and peptide-loaded APCs were cultured as described for “Granule polarization imaging,” except 2 × 10⁵ JR209 T cells and 1 × 10⁶ peptide-loaded T2-A2Kb APCs were imaged for 8 h, acquired at 3-min intervals with a ×40 objective (LD Plan-Neofluar, 0.6 numerical aperture, Zeiss). For analysis, initial T cell/APC conjugation and APC death were manually identified, and T cell/APC conjugates lasting >10 min were analyzed. Cytotoxic efficiency was calculated as (T cell/APC conjugates that result in APC lysis)/(total stable T cell/APC conjugates observed). Image acquisition and analysis were performed using MetaMorph microscopy automation and image analysis software (Molecular Devices).

Calcium flux imaging
 Imaging of T cell calcium flux was performed as previously described (23, 24). T cell and peptide-loaded APCs were cultured and imaged as described for “Granule polarization imaging,” except T cells were treated

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with fura 2-AM dye before combining with peptide-loaded T2-A2Kb APCs. Images were acquired under DIC illumination, as well as detection at 510 nm from both 340 and 380 nm excitation with a ×40 oil objective (EC Plan-NeoFluar, 1.3 numerical aperture, Zeiss). Image acquisition and analysis were performed using MetaMorph microscopy automation and image analysis software (Molecular Devices). The ratio of the fura 2-AM emission intensity at 510 nm resulting from 340 and 380 nm excitation was determined to qualify the relative intracellular calcium concentration. Intracellular calcium concentration is reported as the integrated whole-cell average 340/380 ratio of intensities for 5 min following T cell APC conjugation.

**Western blot analysis**

T cells were lysed in ice-cold lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% n-dodecyl-β-D-maltoside, 1 mM Na3VO4, plus complete protease inhibitor mixture (Roche) and phosphatase inhibitor mixture 2 (Sigma-Aldrich) for 30 min at 4°C, and clarified by centrifugation at 16,000 × g for 15 min at 4°C and collection of supernatant. Lysate was separated by SDS-PAGE, transferred to nitrocellulose membranes, and quantified by near-infrared fluorescence with the Odyssey imaging system (LI-COR Biosciences). For quantification of protein phosphorylation, values were calculated as the intensity of phosphorylated-specific Ab over intensity of total protein-specific Ab, normalized to unity for TCM. For quantification of all other proteins, values were calculated as the intensity of the protein of interest over intensity of actin loading control, normalized to unity for TCM. For phospho-Zap-70 time course experiments, values were calculated as the intensity of phospho-Zap-70 over the intensity of total Zap-70, normalized to time 0 and relative to maximum value attained by either cell type.

For assessment of phosphorylation of Zap-70 after activation, T cells and peptide-loaded T2-A2Kb APCs were combined at a ratio of 2:1, centrifuged briefly to induce conjugation, and incubated at 37°C. At indicated times, ice-cold PBS was added and cells were pelleted and immediately lysed and then assessed by Western blot as described above. For assessment of Cbp phosphorylation, lysates were incubated with anti-Cbp Ab overnight at 4°C, followed by incubation with protein A/G beads for 4 h at 4°C. Beads were washed twice with lysis buffer and once with water prior to addition of phosphate assay buffer. Immunoprecipitation with Cbp–specific Ab was performed exactly as described for Cbp. Blots were probed with both Shp-1– and Themis-specific Abs and quantified as the intensity of Themis over the intensity of Shp-1, normalized to unity for TCM. Calculation of the percentage of Lck phosphorylated at Y394 was performed as described (12). Following clarification, lysates were preclayed by incubation with protein A/G beads (Pierce) for 2 h at 4°C, then incubation with either anti–pY394-Lck or Ab type control IgG overnight at 4°C, followed by depletion of protein–Ab complexes by incubation with protein A/G beads for 4 h at 4°C. Depleted lysate was separated by SDS-PAGE, transferred to nitrocellulose membranes, and quantified by near-infrared fluorescence with the Odyssey imaging system (LI-COR Biosciences). The percentage of Lck that is phosphorylated at Y394 was calculated as: (Lck depleted/pY394 depleted) × 100, where Lck depleted = 1 − (intensity of Lck from pY394 immunoprecipitate (IP)/intensity of Lck from control IP), and pY394 depleted = 1 − (intensity of pY394 from pY394 IP/intensity of pY394 from control IP).

**Quantification of Shp-1 phosphatase activity**

Cells were lysed in buffer containing 50 mM Tris (pH 8), 10 mM EDTA, 150 mM NaCl, and 1% Nonidet P-40 plus protease and phosphatase inhibitors. Clarified lysates were incubated overnight with protein A magnetic beads (Pierce), incubated with 5 μg of an antibody recognising the TCR pairing with the TCR-β chain as per manufacturer’s instructions. The immune complexes were washed twice with lysis buffer without phosphatase inhibitors and twice with phosphatase assay buffer (62 mM HEPES (pH 5), 6.25 mM EDTA, 12.5 mM DTT). Immune complexes were then incubated in 200 μl phosphatase assay buffer plus 25 mM p-nitrophenyl phosphate for 30 min at 30°C under shaking. After centrifugation to removed immune complexes, 800 μl 1 N NaOH was added to the supernatants and the OD was measured at 410 nm. The corresponding immune complexes, bound to protein A–Sepharose, were then analyzed by SDS-PAGE and immunodetection of Shp-1 was performed by Western blotting. Phosphatase activity was calculated by normalizing the OD 410 nm values to the amount of Shp-1 immunoprecipitated, as quantified by Western blot.

**Colocalization analysis**

For in vitro–derived JR209 TCM and TEM, cells were individually (TCM or TEM) cultured on 10% poly-1-lysine–treated coverslips for 30 min at room temperature, fixed with 4% paraformaldehyde for 15 min at room temperature, washed twice with 2% BSA in PBS, and permeabilized with 0.1% Triton X-100 for 7 min at room temperature. Cells were then washed twice and blocked with 5% BSA in PBS for 2 h at room temperature and incubated with primary Abs for Lck and Shp-1 for 1 h at room temperature or overnight at 4°C, washed twice and then incubated with secondary Abs (anti-rabbit IgG F(ab′)2–alkaline phosphatase, anti–mouse IgG F(ab′)2–FITC) for 15 min at 4°C and washed twice. Antifade with DAPI was added and coverslips were mounted on microscope slides. Images were collected at room temperature on a Leica TCS SP II confocal microscope with ×63 oil objective. Leica HC PL APO 1.40 × 0.70 objective using Leica LAS AF software. Colocalization analysis was performed on background-subtracted confocal images using ImageJ software (National Institutes of Health), and the Pearson correlation coefficient (R) was obtained using the JACoP plugin.

For in vivo–derived JR209 TCM and TEM, CD8+-enriched cells were cultured on coverslips as above. Prior to fixation, cells were incubated with PerCP-Cy5.5-conjugated anti-CD44 and PE-conjugated anti-CD62L for 20 min at room temperature. Cells were then washed, fixed, permeabilized, and stained for Lck and Shp-1 as described above. For analysis, cells were first identified as either TCM (CD44+), CD62Llo) or TEM (CD44+, CD62Lhi). The threshold whole cell–integrated PE intensity to distinguish between CD62L high versus low was determined by calculating the percentage of TCM and TEM in similarly stained cells by flow cytometry, and then determining the PE intensity that separated the analyzed cells into similarly proportioned populations. Colocalization analysis was then similarly performed as described above.

**Flow cytometry**

For evaluation of Lck, pY394 Lck, pY505 Lck, Shp-1, and pS591 Shp-1 expression in in vitro–derived JR209 TCM and TEM, lymphocytes were collected from the lymph nodes and spleens of 8- to 10-mo-old mice and CD8+ T cells were purified by negative separation, as described above. T cells were stained with FITC-conjugated anti-CD8, PerCP-Cy5.5-conjugated anti-CD44, and PE-conjugated anti-CD62L Abs prior to being fixed with Fix Buffer I (BD Biosciences) and permeabilized with Perm Buffer III (BD Biosciences) as instructed by the supplier. Cells were then stained with Ag-specific Abs in PBS with 2% FBS, followed by allophycocyanin-conjugated species-appropriate IgG. Samples were collected on an LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo X 10.7 (FlowJo, Ashland, OR) with analysis software (TreeStar). TCM were identified as CD44hi, CD62Lhi and TEM as CD44hi, CD62Llo.

**Markov chain modeling (Lck come&stay/signal duration model)**

The equations governing the Markov chain model were extrapolated from the “Lck come&stay/signal duration” model developed by Chakraborty and colleagues (14). The model assumes an absorbing state once a CD8 molecule bound to an active Lck (Y394) binds to the TCR–pMHC complex. Relevant parameters were taken from literature (14, 25, 26) or experimentally derived in this study. The equations were numerically solved using MATLAB (MathWorks).

**Statistical analysis**

Statistical analysis was performed using a Student t test. A Mann–Whitney U test was performed for single-cell measurements where indicated. Individual cytotoxic efficiency experiments were assessed by a difference in proportions test for independent samples. In all cases, p values <0.05 were considered statistically significant. Error bars represent SEM unless otherwise stated. For box plots, the bottom and top of box represent the first and third quartiles, respectively; internal line represents the second quartile (median); and lower and upper whiskers represent the minimum and maximum data values, respectively.
Results

Inferior CD8\(^\text{+}\) T\(_{CM}\) cytotoxicity is due to an absence of cytotoxic granule delivery in a greater fraction of T cell/APC conjugates compared with T\(_{EM}\).

To address the role of activation signaling affecting the sensitivity of CD8\(^{+}\) memory T cell effector function, we determined the cytotoxic properties of T\(_{CM}\) and T\(_{EM}\) in a well-characterized model system, that is, the humanized TCR-transgenic mouse model JR209, consisting of mouse T cells expressing the human R6C12 α/β TCR variable regions spliced onto mouse constant regions (16), recognizing the anchor-modified melanoma differentiation tissue-associated Ag gp100\(_{209\text{-}217}\) (27) presented by chimeric A*0201/H2K\(^{\text{b}}\) (28). T\(_{CM}\) and T\(_{EM}\) were derived through single-cell imaging to quantify the kinetics of T\(_{CM}\) and T\(_{EM}\) conjugates compared with TEM when cultured with T2-A2Kb APCs loaded with 10 \(\mu\text{M}\) gp100\(_{209\text{-}217}\). T\(_{CM}\) and T\(_{EM}\) were derived through intra Ag experience and exposure to IL-15 and IL-2, respectively (15, 29), achieving CD44, CD25, and CD62L by flow cytometry. (Fig. 1A), which showed that T\(_{EM}\) had significantly higher specific lysis than did T\(_{CM}\), consistent with established trends of CD8\(^{+}\) T\(_{CM}\) and T\(_{EM}\) (7, 8, 33).

Expression of cytotoxic effector molecules correlates with cytotoxic activity of CD8\(^{+}\) T cells (34). We therefore considered that higher T\(_{EM}\)-specific lysis could be due to higher expression and delivery of cytotoxic effector molecules. RT-PCR analysis of granzyme B, perforin, and Fas ligand (Fig. 1C) revealed that T\(_{EM}\) had greater expression of granzyme B compared with T\(_{CM}\), although not for Fas ligand and perforin. It was unclear whether the lower levels of granzyme B in T\(_{CM}\) would be solely responsible for lower specific lysis. Therefore, we quantified the delivery of cytotoxic granules by JR209 T\(_{CM}\) and T\(_{EM}\) by live cell fluorescence microscopy of T cells stained with granule-labeling LysoTracker Red interacting with T2-A2Kb presenting gp100\(_{209\text{-}217}\) (Supplemental Fig. 1A). The time for effector molecules to polarize to the T cell/APC interface following conjugation was similar for T\(_{CM}\) and T\(_{EM}\) (Supplemental Fig. 1B). Notably, in both T\(_{CM}\) and T\(_{EM}\) >95% of T cell/APC conjugates that achieved polarization of effector molecules resulted in APC lysis. Combined, this suggests that lower T\(_{CM}\)-specific lysis may not be due to suboptimal expression or delivery of effector molecules, but rather an absence of effector molecule polarization altogether in a larger fraction of T\(_{CM}\). This may result from insufficient activation signaling in T\(_{CM}\), as granule recruitment and polarization along the centrosome depend on the strength of TCR signal (35).

To explore this idea further, we observed T cell/APC interactions by single-cell imaging to quantify the kinetics of T\(_{CM}\) and T\(_{EM}\) cytotoxicity. Greater cytotoxicity of T\(_{EM}\) could be caused by the ability of individual T\(_{EM}\) to kill multiple target cells, possibly due to greater expression of effector molecules (36). Profiling of the interactions between individual T cells and multiple APCs utilizing time-lapse imaging microscopy in nanowell grids (20, 21) revealed that T\(_{EM}\) were more likely to serially kill APCs (Fig. 1D). Although this could explain T\(_{EM}\) greater overall specific lysis, when we considered T cell interactions with single APCs, T\(_{EM}\) had a significantly higher cytotoxic efficiency (the fraction of stable T cell/APC conjugates that result in APC lysis) than did T\(_{CM}\) (Fig. 1E), suggesting that T\(_{CM}\) are less efficient at inducing APC lysis. Collectively, these results suggest that lower T\(_{CM}\) cytotoxic properties are due to a lesser ability to serially kill APCs, but also a lower efficiency at inducing APC apoptosis upon T cell/APC conjugation. Therefore, differences in cytotoxicity between

![FIGURE 1](http://www.jimmunol.org/ at Univ of Houston on June 7, 2016)
**CD8* T EM have higher levels of activation-induced Zap-70 phosphorylation and calcium signaling compared with T CM**

The magnitude of TCR-proximal signaling induced by TCR ligation correlates with T cell activation and functional performance (12, 39). We considered that the decreased fraction of T CM that achieve cytotoxic effector function compared with T EM might be due to lower TCR-proximal signaling. We quantified activation-induced phosphorylation of Zap-70 following JR209 T CM and T EM incubation with T2-A2Kb presenting gp100209–217(2M) by Western blot analysis (Fig. 2A). T EM achieved higher Zap-70 phosphorylation than did T CM during 5 min of T cell/APC conjugation (Fig. 2B).

We next quantified activation-induced cytoplasmic calcium influx by single-cell imaging of fura 2-AM–labeled T CM and T EM (Fig. 2C), as the quality of upstream TCR signaling quantitatively affects calcium influx (40). T EM attained higher calcium levels than did T CM (Fig. 2D). Notably, for both memory subsets, every stable T cell/APC conjugation resulted in elevation of T cell cytoplasmic calcium at least 70% above baseline levels, with the vast majority of cells reaching >100% above baseline levels (Fig. 2E), although T EM achieved significantly higher maximum levels. Therefore, all observed T CM/APC and T EM/APC conjugates achieved some degree of TCR-dependent signaling, whereas only 61% of T CM induce APC lysis, compared with 91% of T EM (Fig. 1C). This suggests that the degree of activation signaling is insufficient to induce complete cytotoxic effector function in a larger fraction of T CM.

Previous studies have shown that the threshold for APC killing is higher than that for calcium influx (41), such that calcium signaling can still be induced from a stimulus that is too weak to induce APC killing. Therefore, the magnitude of TCR signaling, not its absence, appears to be the source of lower cytotoxic effector function in T CM. Furthermore, based on Zap-70 phosphorylation, the differences in T CM and T EM appear to arise very early in activation signaling.

**CD8* T EM have higher levels of constitutive Lck activity compared with T CM**

The role of Lck as the primary initiator of CD3 ITAM and Zap-70 phosphorylation following TCR ligation is well established (10, 42). Given the differences between T CM and T EM in phosphorylation of Zap-70, we investigated the role of Lck in driving functional differences between T CM and T EM. It has been suggested that the active pool of Lck in resting T cells could be promptly used to initiate signaling following TCR ligation (12). We hypothesized that differential levels of constitutive Lck activity between T CM and T EM could lead to differential signaling following TCR ligation. We compared the phosphorylation of both Lck Y394 and Lck Y505 in resting JR209 T CM and T EM by Western blot analysis, which showed that relative to T CM, T EM had greater levels of pY394 and lower levels of pY505 (Fig. 3A), suggesting that resting T EM may have higher levels of active Lck. We also assessed Lck phosphorylation in T CM and T EM derived in vivo from aged (10–12 mo) JR209 mice (43). Flow cytometry analysis revealed that T EM have higher levels of pY394 and lower levels of pY505 relative to T CM (Fig. 3B). Therefore, in both in vitro–generated and in vivo–derived resting CD8* memory T cells, T EM have higher phosphorylation of activating Lck Y394 and lower phosphorylation of inhibitory Lck Y505 compared with T CM, suggesting that a higher amount of Lck is constitutively active in T EM.

Previous calculations of Lck phosphorylation showed that ~38% of Lck was phosphorylated at Y394 in unsorted naive human CD4* T cells, and ~50% was phosphorylated in immortalized Jurkat cell line (12). To determine how CD8* T CM and T EM...
compare with these cell types, we calculated the amount of pY394 as a percentage of total Lck by immunoprecipitation and Western blot analysis of the resulting depleted lysate (Fig. 3C) (12). In TEM, 54% of Lck is pY394, compared with TCM, in which 18% of Lck is pY394 (Fig. 3D). Although our calculations for JR209 TEM are similar to values previously calculated for naive human CD4+ T cells and Jurkat cells (12), the calculated values for TCM are significantly lower, suggesting that there is heterogeneity in constitutive Lck phosphorylation between these CD8+ memory T cell subsets.

We next determined Lck kinase activity by quantifying the phosphorylation of recombinant CD3ζ-chain by Lck immunoprecipitated from resting JR209 TEM and TCM lysates (12) (Fig. 3E). Correlative to Lck phosphorylation, TEM have higher Lck kinase activity compared with TCM (Fig. 3F). These data combine to suggest that TEM and TCM possess distinct levels of constitutive Lck phosphorylation and activity. As such, TEM may be more poised to initiate signaling following TCR ligation, quantitatively affecting the degree of ITAM and Zap-70 phosphorylation and subsequent signal amplification, and thus the probability of inducing effector molecule polarization and cytotoxic effector function.

To consider the significance of the differences in Lck activity between TCM and TEM, we used a recently published model of T cell activation, the Lck come&stay/signal duration model (14), which posits that coreceptor-mediated delivery of active Lck to the pMHC–TCR complex is the most proximal limiting step in a kinetic proofreading model of T cell activation (44). The probability of forming an active TCR complex is considerably higher for TEM compared with TCM, and it more closely resembles the probability profiles for both Jurkat cells and naive human CD4+ T cells (12) (Fig. 3G, Supplemental Fig. 2). This model suggests that, in a stochastic model of T cell activation (26, 45–47), small changes in the amount of active Lck may significantly affect the probability of formation of a signal-activating pMHC–TCR complex.

Differential regulation of constitutive Lck activity in CD8+ TEM and TCM

To understand the mechanistic basis driving the differences in TCM and TEM Lck activity, we determined the extent of Lck interaction with Shp-1 and Csk, which affect Lck activity by dephosphorylation of Lck Y394 (48) and phosphorylation of Lck Y505 (49), respectively. The cellular localization of Shp-1 and Csk are me-
diated by their interaction with Themis and Cbp, respectively (50). Western blot analysis revealed no significant differences in the expression of Shp-1, Csk, Themis, or Cbp in JR209 TCM and TEM, although Themis showed a trend toward higher expression in TEM (Supplemental Fig. 3A). However, differences in cellular localization of Shp-1 or Csk could result in differential association with, and regulation of, Lck. We determined the colocalization of Lck with Shp-1 and Csk in JR209 TCM and TEM by confocal imaging and calculation of the Pearson coefficient. Both Shp-1 and Csk had higher colocalization with Lck in TCM compared with TEM (Fig. 4A, 4B), suggesting increased interactions of these negative regulatory molecules with Lck in TCM. Colocalization analysis was also performed with in vivo–derived JR209 TCM and TEM, which showed similar results for both Shp-1 and Csk (Fig. 4C), further suggesting that Shp-1 and Csk may have a greater influence on Lck phosphorylation in TCM compared with TEM.

To determine how the interaction of both Csk and Shp-1 with Lck differs between TCM and TEM, we looked at their known interaction partners Cbp and Themis, respectively. Cbp is a membrane protein that brings Csk to the membrane and in closer proximity to Lck in a phosphorylation-dependent manner (50). Immunoprecipitation of Cbp followed by Western blot analysis using a phospho-tyrosine–specific Ab revealed that Cbp was similarly phosphorylated in TCM and TEM (Supplemental Fig. 3B). Shp-1 constitutively interacts with Themis in a GRB2-dependent manner (51). To examine the interaction of Shp-1 with Themis, we immunoprecipitated Shp-1 from TCM and TEM lysate and quantified both Shp-1 and Themis by Western blot analysis (Supplemental Fig. 3C). TCM showed a slightly higher amount of Themis pulled down with Shp-1, although this was not a statistically significant difference from TEM. Therefore, it does not appear that the amount of Shp-1 in complex with Themis, nor the amount of Csk associated to Cbp, accounts for differences in their localization and interaction with Lck between TCM and TEM.

Constitutive Shp-1 S591 phosphorylation causes both decreased phosphatase activity and decreased membrane localization (52), whereas phosphorylation of Y536 and Y564 result in increased phosphatase activity (53). Western blot analysis revealed that JR209 TCM have higher Shp-1 pS591 compared with TCM (Fig. 4D), a trend that was also observed in in vivo–derived JR209 TCM and TEM (Fig. 4E). No differences were observed for Y536 or Y534 (Supplemental Fig. 3D), which supports our observation that Shp-1 complexes with Themis/Grb2 similarly in TCM and TEM, as phosphorylation of both of these sites promotes interaction with Grb2 (54). We next determined Shp-1 phosphatase activity by quantifying the hydrolysis of p-nitrophenyl phosphate to p-nitrophenol by Shp-1 immunoprecipitated from resting JR209 TCM and TEM lysates. Correlative to Shp-1 phosphorylation, TCM have lower Shp-1 phosphatase activity compared with TCM (Fig. 4F). Together with our colocalization data (Fig. 4A–C), this suggests that in TEM Shp-1 has decreased phosphatase activity and membrane localization compared with TCM, and this may contribute to differences in Shp-1 interaction with, and dephosphorylation of, Lck Y394 in resting T cells.

To further address this possibility, we quantified Lck Y394 phosphorylation in resting JR209 TCM and TEM following incubation with the Shp-1 inhibitor NSC-87877. Western blot analysis revealed that NSC-87877 treatment eliminated the differences in Lck pY394 between TCM and TEM (Fig. 4G). Furthermore, analysis of cytotoxicity of TCM and TEM following incubation with NSC-87877 resulted in a significant increase in specific lysis by TCM with minimal increase in TEM-specific lysis over a range of E:T ratios (Fig. 4H). Specifically, at an E:T ratio of 2.5:1, inhibition of Shp-1 resulted in an average increase in specific lysis of 36% for TCM compared with only 7% for TEM. Collectively, these data provide further evidence that Shp-1 contributes to the differences in constitutive Lck activity and effector function between TCM and TEM (55) by disproportionately affecting TCM.

Taken together, these results suggest that constitutive Lck activity, regulated by Shp-1 and Csk, differs between JR209 TCM and TEM contributing to differences in TCR activation signaling and the probability of formation of a signal-activating pMHC–TCR complex.

Discussion

The role of constitutive Lck activity in the relative responsiveness of memory T cell subsets has not been studied. We show that differences in constitutive Lck activity between TCM and TEM result from differential interactions with and activity of Shp-1, and we also suggest a role for Csk. Modeling of TCR-proximal activation signaling suggests that these differences lead to TCM being better poised to initiate TCR-proximal signaling immediately following TCR ligation.

The functional roles TCM and TEM in recall responses are still being clarified, although it is clear that these subsets differ in a number of properties, including expression of surface makers, tissue localization, recall proliferation, cytokine production, and cytotoxic activity (7, 56–59). Our results suggest that TEM cytotoxic superiority may be due to differences in activation signaling and not just expression of effector molecules. However, it is well established that TCM are functionally better than TEM in terms of proliferation and cytokine production, specifically IL-2 (7, 8, 33). Consolidating this with our conclusion that TCM achieve lower levels of TCR signaling and cytotoxic effector function is important to understand the divergent properties and functions of CD8+ T cell memory subsets.

The downstream pathways that drive cytokine production and proliferation are distinct (39), and although proliferative responses show a dependence on the degree of CD3 ITAM phosphorylation, similar cytotoxic responses are initiated with varying degrees of ITAM phosphorylation (39). Determining how the signaling pathway of cytotoxic effector function activation diverges from proliferative and cytokine production pathways will help to understand how TCM and TEM attain their characteristic functional properties.

In this study, we show that Lck activity influences the frequency of TCM versus TEM achieving threshold signaling to induce granule polarization and cytotoxic effector function. Importantly, in nearly all cases where polarization of effector molecules was observed, in both TCM and TEM, APC lysis was observed. Thus, cytotoxic effector function is an all-or-none proposition, such that lower overall levels of cytotoxic function by TCM are due to a greater fraction of TCM/APC conjugates not achieving sufficient signaling to induce effector molecule polarization, compared with TEM. However, higher expression of effector molecules may enable TEM to more effectively serially kill target cells, further contributing to their superior cytotoxic function. Analogous all-or-none results have been reported for recall proliferation of memory T cells, wherein an invariant threshold for the induction of proliferation exists, and the frequency of T cells reaching the signaling threshold was dependent on Ag dose (60). In our study, the distinguishing factor that affects the frequency of TCM versus TEM reaching the signaling threshold to induce cytotoxic effector function when exposed to similar Ag dose is instead Lck activity. Other factors, including Ag dose and TCR affinity, would affect the probability of achieving sufficient signaling to induce effector function (61). Therefore, the extent that constitutive Lck activity affects memory T cell sensitivity may depend on these factors, such that Lck activity may have a diminishing effect on T cell sensitivity with increasing TCR affinity.

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FIGURE 4. Shp-1 mediated differences in Lck activity between TCM and TEM (A) JR209 TCM and TTEM stained for Lck (green), DAPI (blue), and either Shp-1 (top) or Csk (bottom) (red). Original magnification ×63. (B) Pearson correlation coefficient ($R_s$) for Lck–Shp-1 (top) and Lck–Csk (bottom). Representative images in (A) display individually calculated $R_s$ (for Lck–Shp-1: mean values, $T_{CM} = 0.55$, $T_{EM} = 0.44$; $T_{CM}$ $n = 53$, $T_{EM} n = 51$. Mann–Whitney $U$ test, $p = 0.0003$). For Lck-Csk: mean values, $T_{CM} = 0.66$, $T_{EM} = 0.60$; $T_{CM} n = 115$, $T_{EM} n = 123$. Mann–Whitney $U$ test, $p = 0.0004$). (C) $R_s$ for Lck–Shp-1 (top) and Lck–Csk (bottom) in in vivo–generated JR209 TCM and TTEM (for Lck–Shp-1: mean values, $T_{CM} = 0.62$, $T_{EM} = 0.57$; $T_{CM} n = 47$, $T_{EM} n = 17$. Mann–Whitney $U$ test, $p = 0.045$; for Lck-Csk: mean values, $T_{CM} = 0.62$, $T_{EM} = 0.53$; $T_{CM} n = 25$, $T_{EM} n = 8$. Mann–Whitney $U$ test, $p = 0.023$). (D) Western blot analysis of phosphorylated Shp-1 S591 ($n = 4$, two-tailed $t$ test, $p = 0.045$). (E) Flow cytometry quantification of phosphorylated Shp-1 S591 (left) and Shp-1 (right) in in vivo–generated JR209 TCM (light, filled) and TTEM (dark, filled). Unfilled profiles represent secondary Ab only controls for TCM (dashed line) and TTEM (solid line). (F) Analysis of Shp-1 phosphatase activity was determined by immunoprecipitation of Shp-1 and incubation with p-nitrophenyl phosphate, addition of 1 N NaOH, and OD measurement at 410 nm. Phosphatase activity was calculated as OD 410 nm divided by the amount of immunoprecipitated Shp-1, as quantified by Western blot, normalized to unity for TCM ($n = 3$, two-tailed $t$ test, $p = 0.013$). (G) Western blot analysis of pLck Y394 following overnight culture with indicated concentrations of Shp-1 inhibitor NSC-87877 (for 0 μM NSC-87877, both $T_{CM} n = 13$; for 50 μM NSC-87877, both $T_{CM}$ and $T_{EM} n = 6$; for 0 μM NSC-87877, two-tailed $t$ test, $p = 0.007$; for 50 μM NSC-87877, two-tailed $t$ test, $p = 0.63$). (H) Cytotoxic properties of JR209 TCM (left) and TTEM (right) toward T2-A2Kb APCs loaded with 10 μM gp100209–217(2M) at E:T ratios indicated, following overnight culture with (solid line) or without (dashed line) 50 μM Shp-1 inhibitor NSC-87877. Plot is representative of triplicate experiments. Specific lysis was calculated as described in Materials and Methods. (I) The percentage changes in specific lysis of $T_{CM}$ and $T_{EM}$ following treatment with Shp-1 inhibitor NSC-87877 toward T2-A2Kb APCs loaded with 10 μM gp100209–217(2M) at 2.5:1 E:T ratio following overnight culture with 50 μM Shp-1 inhibitor NSC-87877. Specific lysis was calculated as described in Materials and Methods ($n = 3$, two-tailed $t$ test, $p = 0.029$). *$p < 0.05$, ***$p < 0.001$.

A great deal is understood about the complex T cell activation signaling network, yet a universal model of T cell activation that accounts for its sensitivity and selectivity remains elusive (62). The Lck come&stay/signal duration model (14) improves upon the kinetic proofreading model (44), which posits that following ligation the TCR complex must undergo a series of steps prior to initiation of downstream activation signaling, enabling discrimination between Ags with small affinity differences. Significantly, the model predicts that coreceptor-mediated Lck recruitment to the pMHC–TCR complex is the most proximal limiting step in this process, and it predicts that the degree of coupling between active Lck and coreceptor strongly influences the probability of active Lck recruitment to the pMHC–TCR complex (14). Accordingly, evaluating the differences in Lck activity between $T_{CM}$ and $T_{EM}$ with this model predicts $T_{EM}$ to have an increased probability of forming an active TCR complex following ligation compared with $T_{CM}$ (Fig. 3G). T cell activation models such as the Lck come&stay/signal duration model (14) presume that Lck activity is not significantly altered following TCR ligation, which suggests that constitutive Lck activity would be an important factor in the initiation of downstream activation signaling (14), and differences in levels of Lck activity between $T_{CM}$ and $T_{EM}$ would be sufficient to alter the probabilities of achieving threshold signaling for T cell activation. This is supported by recent work by Manz et al. (13), which used titration of a Csk-specific inhibitor to induce a 3- to 4-fold increase in Lck Y394 phosphorylation in CD8$^+$ T cells. Importantly, whereas increased Lck activity led to weak phosphorylation of signaling molecules downstream of

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Zap-70, coupling this with TCR stimulation led to enhanced downstream phosphorylation (13). Their work therefore suggests that small changes in the amount of active Lck (as little as a 50% increase) can significantly affect the magnitude of activation signaling following TCR ligation. In our study, TEM have significantly higher Lck Y394 phosphorylation than do TCM (54% of Lck molecules compared with 18% for TCM, Fig. 3C, 3D) and, correspondingly, TEM have a roughly 60% higher kinase activity (Fig. 3E, 3F), and would thus strongly influence the magnitude of activation signaling and the probability of achieving threshold levels of signaling to induce effector function.

Our results also show that TCM and TEM differ in Lck Y505 phosphorylation (Supplemental Fig. 3A, 3B), which may contribute to the overall differences in Lck activity between TEM and TCM (Fig. 3C, 3D). However, the inhibitory effect of Lck Y505 phosphorylation on Lck activity is overcome by simultaneous Lck Y394 phosphorylation (12). It is possible that higher levels of Lck Y505 phosphorylation in TEM may actually decrease the rate of trans-autophosphorylation of Y394 (63) and therefore contribute to the lower levels of Lck pY394 observed in TCM. Our observation that Csk and Lck are more colocalized in TCM compared with TCM may explain why TEM have higher levels of Lck pY505. It has previously been shown that Csk is differentially distributed in naïve versus Ag-experienced CD8+ T cells, affecting Csk colocalization with Lck (64). The mechanism behind the differential localization of Csk is unclear, although evidence suggests that Lck activity may not be required for Csk interaction and to which Shp-1 can dephosphorylate Lck Y394, which may result in greater sequestration from Csk. Therefore, higher levels of Lck pY394 in TEM, driven by differences in Shp-1 activity, could lead to greater clustering of Lck and resulting lower levels of Lck pY505 owing to decreased membrane interaction with Csk. However, it must be considered that other mechanisms may contribute to the differential colocalization of Csk with Lck.

Shp-1 has been shown to interact constitutively with Themis in a GRB2-dependent manner, and this complex associates with phosphorylated LAT to mediate negative feedback to dampen downstream signaling (51). Interestingly, Paster et al. (51) showed that knocking down Themis expression had no effect of constitutive Lck activity, although it did have a significant effect on TCR-proximal signaling, presumably due to decreased recruitment of Shp-1 to LAT. Our results showing the importance of Shp-1 in establishing the differences in constitutive Lck activity between TCM and TEM suggest therefore that this is mediated by Shp-1 in a Themis-independent manner. Our data suggest that differences in Shp-1 S591 phosphorylation between TCM and TEM result in differential Shp-1 membrane localization and phosphatase activity, which affects the degree to which Shp-1 can interact with Lck and to which Shp-1 can dephosphorylate Lck Y394, respectively (52). Although basal Shp-1 serine phosphorylation has been observed in resting T cells, the mechanism controlling Shp-1 S591 phosphorylation remains unclear (52). Although protein kinase C has been implicated in mediating Shp-1 S591 phosphorylation (66), other recent evidence does not support a role for protein kinase C in Shp-1 S591 phosphorylation, or at the very least suggests that other basophilic kinases have a role (52). Thus, further investigation is warranted into the mechanism by which constitutive Shp-1 phosphatase activity is controlled in resting memory T cells and how this directly affects constitutive Lck activity.

Although it has been shown that Lck activity is not required for maintenance, in vitro function, and secondary activation in vivo of virus-specific CD8+ memory T cell (67), our results suggest that Lck activity may be important in determining the sensitivity of self-antigen–specific CD8+ memory T cells. The contribution of constitutive Lck activity to CD8+ memory T cell sensitivity may therefore be dependent on the Ag. For example, the virus-specific memory responses induced by gp33-specific TCR-expressing T cells (Kd = 3 μM) (68) are independent of Lck activity (67). However, responses to lower affinity self-antigens, such as gp100 (Kd = 9.3 μM) (69), as described herein, may be Lck-dependent. Alternatively, it is possible that Lck activity may not be required for JR209 TCM and TEM to elicit responses to gp100, but that the presence of Lck confers a further degree of sensitivity and enables more robust and/or selective responses to self-antigens. A more comprehensive understanding of the role of Lck in memory T cell responses will contribute to the overall understanding of how T cell sensitivity is established at the most proximal stages of TCR signaling.

Our findings also provide insight into further optimization of the in vitro preparation of T cells for use in clinical adoptive cell therapy applications (70) to produce effective in vivo antitumor properties, including effective homing, proliferation, persistence, and target cell lysis. In a number of experimental systems, TEM show superior antitumor responses compared with TCM (7, 15, 71), despite lesser cytotoxic effector properties (8). Therefore, it is possible that TEM antitumor responses may be improved further by targeting TCR-proximal signaling components that drive cytotoxic effector function sensitivity, including Shp-1. Taken together, understanding the contributions of T cell maturation state, TCR affinity/avidity, and activation signaling components to T cell effector function sensitivity will help to guide the selection and manipulation of the optimal antitumor T cells for adoptive immunotherapy. Our finding that differences in constitutive Lck activity between TCM and TEM lead to TEM being better poised to initiate TCR-proximal signaling represents a mechanism by which altered preligation activation states of TCR-proximal signaling components can affect T cell activation sensitivity. As such, Lck may represent a potential target for modification of T cell sensitivity for targeted T cell therapies for cancer and autoimmune diseases.

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Disclosures

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