

# Lck Regulates the Threshold of Activation in Primary T Cells, While both Lck and Fyn Contribute to the Magnitude of the Extracellular Signal-Related Kinase Response<sup>∇</sup>

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**The src family kinases p56<sup>lck</sup> (Lck) and p59<sup>fyn</sup> (Fyn) are the most proximal signaling molecules to be activated downstream of the T-cell receptor. Using an inducible transgenic model, we can regulate the expression of Lck in primary T cells and ask how the signaling cascade and differentiation potential are affected by the absence or the presence of reduced levels of Lck. We show that in naïve T cells, Lck controls the threshold of activation by preferentially regulating multiple signaling pathways that result in the mobilization of Ca<sup>2+</sup> through activation of phospholipase C-gamma and protein kinase C as well as activation of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway. Fyn is also able to stimulate the ERK/MAPK pathway in primary T cells but has little influence on the mobilization of Ca<sup>2+</sup>. Only Lck efficiently stimulates production of diacylglycerol and therefore RasGRP1 recruitment to the plasma membrane and phosphorylation of Shc, suggesting that Fyn activates ERK via a different upstream signaling route. Finally, we show that signals through Lck are essential for the development of T-cell-effector potential, particularly for effective cytokine transcription.**

Signaling through the T-cell receptor (TCR) can result in positive or negative selection of immature thymocytes and activation or anergy of mature T cells. The biochemical pathways that trigger these opposed outcomes are not well understood. The most proximal signaling molecules to be activated upon TCR engagement are the Src family kinases, p56<sup>lck</sup> (Lck) and p59<sup>fyn</sup> (Fyn), and genetic and biochemical studies have shown them to be essential for signaling through the TCR/CD3 complex (17). Moreover, altering Lck availability has been shown to influence cell fate (18, 26, 55), indicating that the activation of these proximal signaling molecules is central in determining the outcome of TCR engagement.

Following TCR engagement, Lck and/or Fyn phosphorylates immunoreceptor tyrosine-based activation motifs (ITAMs) in CD3 and TCR $\zeta$  (45, 49), enabling ZAP-70 to bind (6). In addition, Lck and Fyn contribute to ZAP-70 activation, promoting recruitment of downstream adaptor molecules and kinases, which initiates a signaling cascade required for proliferation and differentiation of both developing thymocytes and mature T cells (17). In Lck<sup>-/-</sup> thymocytes, the basal level of TCR $\zeta$  phosphorylation is low and does not increase substantially after TCR cross-linking and, furthermore, no ZAP-70 phosphorylation can be detected (49). Similarly, Lck<sup>-/-</sup> cell

lines show profound defects in early TCR activation and fail to mobilize Ca<sup>2+</sup> in response to TCR triggering (2, 8, 22, 45). However, little is known about how lack of Lck influences the response of primary peripheral T cells, as the production of mature lymphocytes is profoundly compromised in Lck<sup>-/-</sup> mice (32). One report used mice expressing an Lck transgene solely in the thymus, to overcome the developmental block associated with the Lck<sup>-/-</sup> background, and showed that primary Lck<sup>-/-</sup> T cells have an impairment in Ca<sup>2+</sup> mobilization and defective proliferation (47). However, these authors also showed that T cells expressing an excess of a catalytic, inactive Lck were capable of fluxing Ca<sup>2+</sup>, suggesting that Lck kinase activity is not essential to its ability to mobilize intracellular Ca<sup>2+</sup>. Therefore, many questions still remain about how Lck interfaces with downstream signaling components of primary lymphocytes.

Although both Lck and Fyn are expressed throughout the T-cell lineage, the relative levels of these two kinases vary during differentiation (33). Moreover, Lck and Fyn function both as kinases and as adapter proteins, through interactions via their SH2 and SH3 domains, which have been shown to interact with overlapping as well as unique target proteins within the cell (57). Analyses of cells from Lck- and Fyn-deficient mice and of cell lines expressing only one or the other kinase (4, 9, 44, 45, 47, 48, 50) have indicated that both of these kinases function as amplifiers of TCR-mediated signaling, increasing sensitivity to stimulation and potentially influencing the differentiation outcome after TCR triggering. However, recent data have suggested that Fyn may also play a role as a negative regulator of T-cell responses, as it interacts specifically with molecules such as the adapter PAG, which may influence the activation of the src kinases themselves (56), and

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at 37°C. Cells were incubated with 10 µg/ml of each biotinylated anti-CD3 and anti-CD4 together with anti-CD8-phycoerythrin and anti-CD44-fluorescein isothiocyanate for 15 min at room temperature. Cells were washed and acquired on an LSR (Becton Dickinson) for 30 s before cross-linking anti-CD3 and anti-CD4 molecules with 20 µg/ml avidin. Data were analyzed using FlowJo (Treestar) software gating on CD8<sup>-</sup> CD44<sup>lo</sup> lymphocytes.

**In vitro stimulations and IL-2 production assays.** CD4 T cells were purified by including anti-CD25 Ab in the depletion. Cells were incubated with 5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) for 10 min at room temperature in prewarmed phosphate-buffered saline and washed once. Triplicate cultures at  $2 \times 10^5$  cells per well were set up with soluble anti-CD3 (as indicated in Fig. 1) plus anti-CD28 at 1 µg/ml in the presence of antigen-presenting cells ( $1 \times 10^4$  bone marrow-derived dendritic cells or  $1 \times 10^4$  Rag-1<sup>-/-</sup> splenocytes). Alternatively, cells were stimulated with 10 ng/ml 12,13-phorbol dibutyrate (PDBu) and 100 nM ionomycin (Sigma). After 72 h, cells were stained with anti-CD4 plus anti-CD5 (53-7.3; Pharmingen) and analyzed on a FACSCalibur using Flow Jo (Treestar) software. Proliferation of CFSE-labeled cells was calculated by determining the frequency (*F*) of cells that had undergone different numbers of division (*d*). Adjusted frequencies (AdF) were calculated by dividing *F* by *2d*. The percentage of cells triggered to divide was calculated by the equation  $1 - (\text{AdF}_0/\Sigma\text{AdF}) \times 100$ . Alternatively, purified CD4 T cells were stimulated with the above-indicated Ab for 24 h. Cells were stained with anti-CD4, anti-CD5, and anti-CD25 Abs. Supernatants were harvested and used in an alamar blue-based cytotoxic T-lymphocyte line 2 assay. Results are percentages of the maximum value for supernatant diluted 1 in 3. TCR and CD69 (H1.2F3; Pharmingen) levels were determined 18 h after stimulation of total LN with 1 µg/ml anti-CD3 and anti-CD28 Abs.

**RESULTS**

**Inducible Lck is upregulated following TCR stimulation.**

We have previously reported mice in which expression of Lck is controlled by the induction of a tet-responsive transgene (26). These mice are on an Lck<sup>-/-</sup> background, and induction of transgene expression with DOX successfully overcomes the lack of endogenous Lck and restores normal thymopoiesis (26). Withdrawal of the mice from DOX for at least 7 days (Lck1<sup>OFF</sup>) is sufficient to remove all residual protein (38). The periphery of DOX-fed, Lck<sup>ON</sup>, and Lck<sup>OFF</sup> mice is populated with near-normal T-cell numbers, although there is an increase in the ratio of CD4<sup>+</sup> to CD8<sup>+</sup> T cells relative to that for WT mice (Fig. 1A). The phenotype of naïve peripheral T cells is normal, with the exception of the level of CD4 on the cell surface, which is clearly lower and correlates with reduced expression of Lck from the transgene in peripheral T cells even when mice are maintained on DOX (38).

As the pool of Lck associated with the coreceptor CD4 is the most relevant for initiation of the response in primary T cells, we checked whether Lck found in Lck<sup>ON</sup> mice is CD4 associated. In contrast to thymocytes, CD4 immunoprecipitates from Lck1<sup>ON</sup> LN T cells showed a 40- to 50-fold reduction of CD4-associated Lck and an overall 10-fold reduction in total intracellular Lck compared to that in WT cells (Fig. 1B) (40). Despite this, peripheral T cells from Lck1<sup>ON</sup> mice respond comparably after antigen or anti-TCR stimulation, although with a delay (38), so we asked whether expression of the Lck transgene altered as the cells became metabolically active (Fig. 1C). Ex vivo cells from Lck<sup>ON</sup> mice in this experiment had less Lck than was usually seen (compare Fig. 1B), probably due to contamination of irrelevant cells, which would overrepresent the actin loading control. However, following stimulation with anti-CD3 and anti-CD28 in the presence of DOX, intracellular Lck was upregulated to within twofold compared to WT levels at 48 h. In addition, a slower-migrating species of Lck that most likely represents the species resulting from phosphoryla-

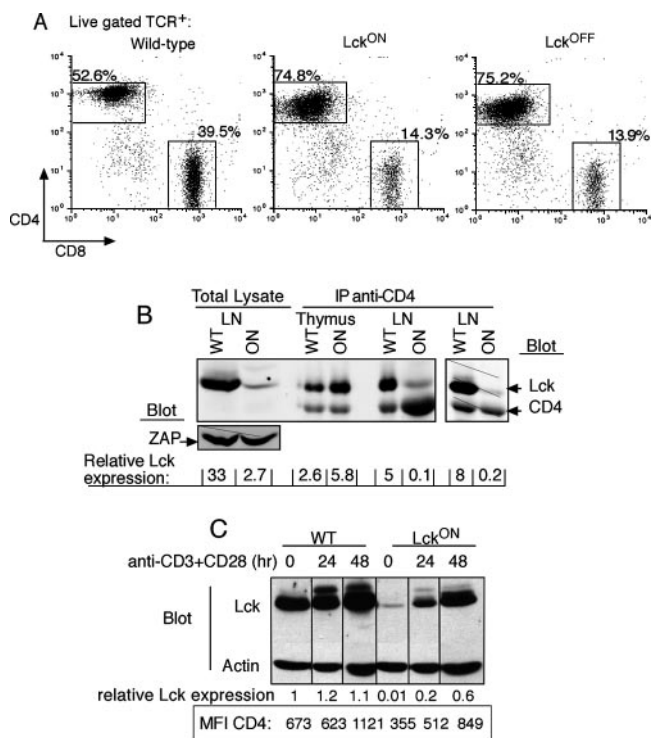


FIG. 1. Expression of the Lck1 transgene in Lck1<sup>ind</sup> mice is up-regulated following activation. (A) CD4 and CD8 expression on gated live TCR<sup>hi</sup> cells from LNs of WT and Lck1<sup>ind</sup> mice on DOX (Lck<sup>ON</sup>) or off DOX (Lck<sup>OFF</sup>) (1 to 2 weeks). (B) Cell lysates were prepared from LNs and analyzed by Western blot analysis for Lck and ZAP-70 as a loading control. CD4 was immunoprecipitated from the thymus and LN, and blots were probed for Lck and CD4. Relative Lck expression levels were determined by normalizing data to those for ZAP-70 or CD4. As the CD4-associated Lck was greatly reduced in Lck<sup>ind</sup> mice, samples were loaded to maximize the Lck signal, and the right panel shows the loading controls for immunoprecipitates from equivalent cell numbers. Quantitation was by infrared imaging using the Li-Cor Odyssey system. (C) LN cells were stimulated with 1 µg/ml CD3 and 1 µg/ml CD28 for 24 and 48 h. Cell lysates were blotted with anti-Lck and antiactin and visualized by chemiluminescence. Relative Lck expression levels were normalized to data for actin, and the unstimulated WT value was set to 1. The CD4 MFIs are presented for comparison.

tion of ser-59 by MAPKs (43, 53) was detected following TCR stimulation. Concomitant with Lck upregulation, expression of CD4 was also increased, as shown by an >2-fold increase in the mean fluorescence intensity (MFI) of CD4 relative to that of ex vivo CD4 T cells at 48 h.

Using peripheral T cells from these mice, we could examine the biochemical outcome of TCR triggering in the absence of Lck as well as in the presence of low levels (1 to 20%) of the kinase by using ex vivo T cells from Lck<sup>ON</sup> mice. Furthermore, we specifically addressed the relevance of coreceptor-associated Lck by comparing the stimulation of primary CD4 T cells with either cross-linked anti-CD3 alone or anti-CD3 plus anti-CD4.

**Lck is required for Ca<sup>2+</sup> responses.** It has been shown previously that T cells lacking Lck are defective in Ca<sup>2+</sup> mobilization after stimulation (45, 47); therefore, we asked whether this was also true for CD4<sup>+</sup> T cells from our inducible

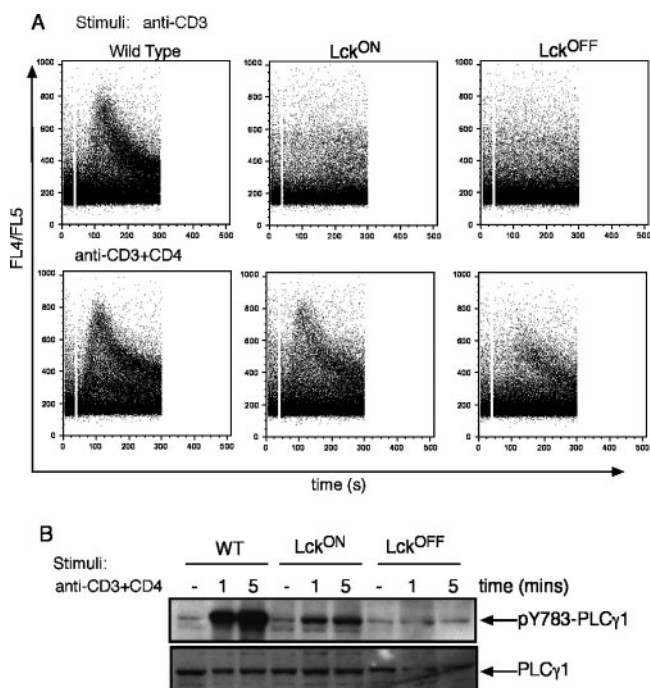


FIG. 2. Defective  $\text{Ca}^{2+}$  and PLC- $\gamma$ 1 phosphorylation in  $\text{Lck}^{\text{OFF}}$  T cells. (A) LN cells were loaded with indo-1 and stimulated with cross-linked anti-CD3 or anti-CD3 plus anti-CD4. FACS plots show the ratios of indo-1 fluorescence against time for CD4 cells. (B) Purified  $\text{CD4}^+$  T cells were stimulated as shown and blotted with anti-PLC- $\gamma$ 1 pY783 or total anti-PLC- $\gamma$ 1 for loading and visualized by chemiluminescence. Data shown are representative of at least three experiments. Percent relative expression was calculated by densitometric analysis of bands and normalizing data for individual phospho bands to those for their loading controls, and the maximal signal was set to 100%.

mice expressing either low levels or no Lck. T cells from WT,  $\text{Lck}^{\text{ON}}$ , and  $\text{Lck}^{\text{OFF}}$  mice were loaded with indo-1, and the  $\text{Ca}^{2+}$  flux was measured after stimulation with cross-linked anti-CD3 or anti-CD3 plus anti-CD4 (Fig. 2A). WT T cells produced robust  $\text{Ca}^{2+}$  fluxes with either stimulus; however, CD3 alone was unable to produce a detectable flux in cells expressing either reduced levels of Lck ( $\text{Lck}^{\text{ON}}$ ) or no Lck ( $\text{Lck}^{\text{OFF}}$ ). When CD4 was cross-linked with CD3, the  $\text{Ca}^{2+}$  flux of T cells from  $\text{Lck}^{\text{ON}}$  mice was restored, presumably because the remaining Lck molecules were more efficiently targeted to the TCR through their association with CD4. Somewhat surprisingly, CD3-plus-CD4 cross-linking, but not CD3 alone, also induced a detectable  $\text{Ca}^{2+}$  response in cells which lacked Lck. The initial  $\text{Ca}^{2+}$  spike was absent; however, a proportion of cells fluxing  $\text{Ca}^{2+}$  was detected at  $>100$  s in  $\text{Lck}^{\text{OFF}}$  cells. It should be noted that the ability of  $\text{Lck}^{-/-}$  CD4 T cells to mobilize  $\text{Ca}^{2+}$  is not due to residual Lck expression, as the same results were obtained with cells from mice withdrawn from the DOX diet for  $>1$  month. These data suggest that CD4 can contribute to T-cell signaling independently of its association with Lck, perhaps by recruiting other signaling molecules, such as the recently described adapter LIME (5, 20).

The mobilization of  $\text{Ca}^{2+}$  in T cells has been shown to be downstream of the activation of PLC- $\gamma$ 1 (54), and Lck, ZAP-70, and ITK have been shown to be important for full PLC- $\gamma$ 1

activation (30). Furthermore, spatial localization of PLC- $\gamma$ 1 through its ability to associate with both LAT and Slp-76 is critical for its function (27). TCR engagement causes both the phosphorylation and the increased catalytic activity of PLC- $\gamma$ 1. Specifically, phosphorylation of Y783 of PLC- $\gamma$ 1 has been shown to be essential for coupling the TCR to IL-2 promoter activity in Jurkat T cells (21). We showed that phosphorylation of PLC- $\gamma$ 1 Y783 correlates directly with expression of Lck (Fig. 2B), with only background bands observed after stimulation of peripheral T cells in the absence of Lck and  $\sim 20\%$  of the WT signal visible when low levels of Lck are available ( $\text{Lck}^{\text{ON}}$  cells). Therefore, there is good correspondence between the requirement for Lck to activate PLC- $\gamma$ 1 and to initiate a  $\text{Ca}^{2+}$  flux after stimulation.

**Defective signaling to ZAP-70 and LAT in the absence of Lck.** The initial substrates to be phosphorylated after TCR triggering are the ITAM motifs on the TCR $\zeta$  and CD3 chains. In resting primary T cells *ex vivo*, there is basal phosphorylation of TCR $\zeta$ , indicative of the cells receiving TCR-associated survival signals. We showed previously that, in the absence of Lck, Fyn maintains the level of the p21 $\zeta$  *in vivo* (40), indicating that either of these Src kinases is capable of phosphorylating the TCR $\zeta$  chain. In order to address the requirement for Lck to induce the hyperphosphorylated 23-kDa form of the TCR $\zeta$  chain after activation, purified  $\text{CD4}^+$  T cells were stimulated with cross-linked CD3 or CD3 plus CD4. In contrast to what was found for WT cells, inducible p23 $\zeta$  was barely detectable after stimulation if Lck was reduced or absent, although constitutive p21 $\zeta$  was seen in all samples (Fig. 3A). This was true regardless of whether the cells were stimulated with anti-CD3 alone or anti-CD3 plus anti-CD4, regardless of the fact that inclusion of anti-CD4 Abs greatly enhanced the production of p23 $\zeta$  in WT cells.

Phosphorylation of the TCR-associated ITAMs allows recruitment of the ZAP-70 PTK and its activation. Measurement of total Tyr phosphorylation from ZAP-70 immunoprecipitates after stimulation revealed that while pZAP-70 could be detected from WT cells stimulated with CD3 plus CD4, none was detectable in immunoprecipitates from  $\text{Lck}^{\text{ON}}$  or  $\text{Lck}^{\text{OFF}}$  T cells (data not shown). However, improved detection of pZAP-70 was achieved with antisera specific for pY319, which is known to be required for the positive regulation of ZAP-70 (10, 52). As seen for phosphorylation of TCR $\zeta$ , there was a profound dependence on the presence of Lck for phosphorylation of Y319 of ZAP-70 (Fig. 3B,  $\text{Lck}^{\text{ON}}$ ). Furthermore, cells expressing low levels of Lck (Fig. 3B,  $\text{Lck}^{\text{ON}}$ ) required cross-linking of CD4 with CD3 before pY319 was detected, and CD3-plus-CD4 cross-linking greatly increased pZAP-70 even in WT cells. The enhancement of signaling was specific for CD4 cross-linked with CD3, as cross-linking CD3 with Thy-1 in WT cells showed no enhancement of ZAP-70 phosphorylation compared to anti-CD3 alone (Fig. 3C). Together, these results indicate that Lck is the main kinase for the phosphorylation of this residue on ZAP-70 and that the physical juxtaposition of the TCR with CD4-associated Lck greatly enhances this activity. Tyrosine phosphorylation of ZAP-70 is known to increase kinase activity, and ZAP-70 Y319 mutants have decreased catalytic activities (10). We asked whether  $\text{Lck}^{-/-}$  T cells show any ZAP-70 kinase activity, by immunoprecipitating ZAP 70 from  $\text{CD4}^+$  T cells stimulated with anti-

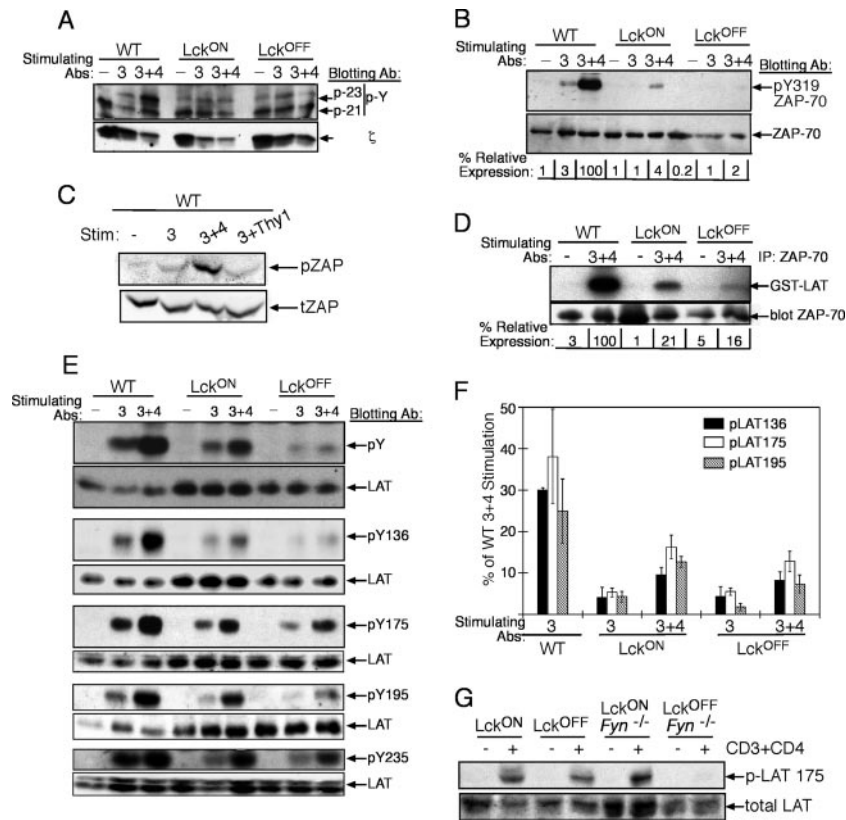


FIG. 3. Proximal TCR signals are defective in the absence of Lck. CD4 T cells were stimulated with anti-CD3, anti-CD3 plus anti-CD4, or anti-CD3 plus anti-Thy1, as shown, for 2 min. Total cell lysates were run on SDS-PAGE gels, immunoblotted for TCRζ chain phosphorylation with anti-pY and reprobed for TCR-ζ (A), and immunoblotted for pY319-ZAP-70 (pZAP) and total ZAP-70 (tZAP) (B and C). (D) ZAP-70 kinase activity from ZAP immunoprecipitates was measured by <sup>32</sup>P incorporation in vitro on GST-LAT substrate. Total ZAP-70 indicates loading. (E) Immunoblots probed with phospho-specific LAT Abs for pY136, pY175, pY195, and pY235 and those shown in panels A to D were visualized with chemiluminescence. (F) Graphs quantifying individual phospho-LAT residues either from the densitometries of ECL blots or directly from a Li-Cor Odyssey infrared imager for three to five replicate experiments are normalized to data for their loading controls and expressed as percentages of the maximum stimulation level, set to 100% based on WT cells stimulated with CD3 plus CD4. (G) T cells expressing Lck plus Fyn (Lck<sup>ON</sup>), Fyn only (Lck<sup>OFF</sup>), Lck only (Lck<sup>ON</sup> Fyn<sup>-/-</sup>), or neither kinase (Lck<sup>OFF</sup> Fyn<sup>-/-</sup>) were stimulated as described above and lysates probed for pLAT 175 and total LAT to control for loading and visualized with a Li-Cor infrared imager.

CD3 plus anti-CD4 Abs and examining the phosphorylation of an exogenous substrate in vitro. For these experiments, we chose a recombinant LAT-GST fusion protein as the most physiologically relevant ZAP-70 substrate. As shown in Fig. 3D, there was small but detectable ZAP-70 kinase activity after CD3-plus-CD4 stimulation even in the absence of Lck. Therefore, while Lck is required for optimal activation of ZAP-70, it is clear that some residual kinase activity can be stimulated in the absence of Lck. It is most likely that Fyn was responsible for this activity, as we found comparable levels of Fyn protein in T cells from all of these mouse strains and all contained equivalent pools of Fyn in the active state, as judged by the phosphorylation of the active residue, Y417 (data not shown).

To address whether the ZAP-70 kinase activity that we detected in vitro was relevant for downstream signaling cascades in vivo, we assessed LAT phosphorylation after stimulation in CD4 T cells lacking Lck. TCR-induced phosphorylation of LAT was detected initially by probing total cell lysates with Ab to pY. A protein that migrated at the same size as LAT could be detected in WT cells stimulated with anti-CD3 as well as anti-CD3 plus anti-CD4 (Fig. 3E, top panel). As with pζ and

pZAP-70, the induction of pLAT by CD3 plus CD4 was much better than that by CD3 alone for both WT and especially Lck<sup>ON</sup> CD4 T cells. Consistent with the weak ZAP-70 kinase activity that was detected in Lck<sup>OFF</sup> cells, a small amount of pLAT activation could also be observed in Lck<sup>-/-</sup> cells, particularly after stimulation with anti-CD3 plus anti-CD4 Abs.

LAT contains nine Tyr residues which when phosphorylated are thought to allow other proteins to bind via their SH2 domains (29). The distal four residues, Y136, Y175, Y195, and Y235, have been shown to be crucial for linking LAT to the activation of both PLC-γ1 and the Ras/ERK pathways in Jurkat T cells (59). Furthermore, knock-in mutant mice in which these four residues are mutated resemble LAT<sup>-/-</sup> mice with thymocyte development arrested at the double-negative stage and no mature T cells (41). More specifically, LAT Y136 has been shown to be important for PLC-γ1 recruitment, thus linking LAT to activation of the Ca<sup>2+</sup> pathway, while Y175, Y195, and Y235 interact with Grb2 and GADS (28, 34, 59). Using antisera specific for individual pYs on LAT, even in the absence of Lck, we could readily detect the phosphorylation of residues Y175 and Y235 but much weaker phosphorylation on

residues Y136 and Y195 (Fig. 3E). In fact, normalization of the data showed that the patterns of phosphorylation levels for residues Y136, Y175, and Y195 were similar between the different cell types, with the highest signal seen with pY175 and the weakest with Y136 (Fig. 3F). At this point, we cannot distinguish whether this reflects differences in the efficiencies with which these individual residues are targets for phosphorylation or simply disparities in the potencies of the specific anti-phospho antisera. However, there remains a direct correlation between the reduced ability of these cells to activate PLC- $\gamma$ 1 and the poor phosphorylation of Y136 when Lck is reduced or absent. As before, the induction of LAT phosphorylation was considerably improved by cross-linking CD4 plus CD3 compared to CD3 alone, even in the absence of Lck; however, it is clear that the optimal phosphorylation of these downstream kinases and adaptor molecules occurred when Lck was activated.

We asked whether Fyn was responsible for the LAT Y175 phosphorylation in the absence of Lck by stimulating cells from Lck<sup>ind</sup> mice crossed to a Fyn<sup>-/-</sup> background. Switching off Lck in these mice produces T cells lacking both kinases, and upon stimulation with anti-CD3 plus anti-CD4, such cells showed no detectable phosphorylation of LAT residues (Fig. 3G), confirming that Fyn can initiate limited signaling downstream of the TCR in the absence of Lck.

**ERK phosphorylation in Lck<sup>-/-</sup> T cells.** In view of the defects in activation that we saw among upstream kinases and adapters in Lck<sup>-/-</sup> cells, we looked further downstream at the activation of the MEK/ERK MAPK pathway. Surprisingly, we found a much smaller reduction in the activation of MEK or ERKs in the absence of Lck by Western blotting than we observed for other upstream signaling molecules. Robust phosphorylation of MEK was detected in the absence of Lck (Fig. 4A), as was phosphorylation of ERK1 and ERK2 (Fig. 4B). Quantitation of several Western blots indicated that levels of ERK phosphorylation in the absence of Lck were consistently in the order of 10 to 30% of those found in WT cells, which contrasts with a reduction of >95% in the phosphorylation of upstream kinases, such as ZAP-70 and PLC- $\gamma$ 1. Moreover, unlike activation of ZAP-70, PLC- $\gamma$ 1, or LAT, inducible ERK phosphorylation was remarkably similar in Lck-sufficient cells stimulated with either anti-CD3 alone or anti-CD3 plus anti-CD4 Ab, further indicating that in WT cells, recruitment of Lck via CD4 has little additive effect on ERK activity. Furthermore, we could confirm that the residual ERK activity in the absence of Lck was primarily mediated via Fyn, as the inducible signal was lost in cells lacking both Lck and Fyn (Fig. 4C).

The ability of Lck<sup>-/-</sup> T cells to activate the ERK pathway was surprising in view of the profound defects in the activation of upstream kinases and adapter molecules and suggested that Fyn could activate the ERK pathway independently of Lck. In order to better understand how these two Src kinases activated the ERK pathway, we moved to a fluorescence-activated cell sorter (FACS)-based assay to monitor ERK activation at a single-cell level. In addition, we looked at an antigen-specific response by using Lck<sup>ind</sup> and Fyn<sup>-/-</sup> mice that were crossed with the F5 TCR transgene on a Rag1<sup>-/-</sup> background. Upon peptide stimulation, F5 CD8 T cells behaved comparably to CD4 T cells by Western blot analysis, in that they showed profound defects in the phosphorylation of upstream kinases,

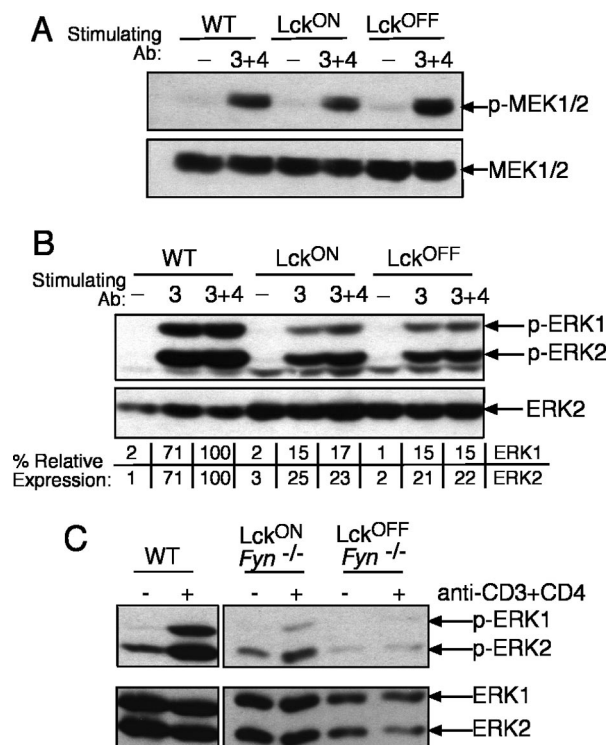


FIG. 4. Efficient MEK and ERK activation in the absence of Lck. pMEK1/2 (A) or pERK1/2 (B) is shown for purified CD4 T cells stimulated for 2 min at 37°C. pERK activation was mediated via Fyn, as no inducible signal was found in cells which express neither Lck nor Fyn (C). Loading was assessed by probing with specific antisera for total protein and visualized with chemiluminescence. Each blot is representative of two to four replicates, and percent relative expression was determined as for Fig. 2.

such as ZAP-70 and PLC- $\gamma$ 1, and in Ca<sup>2+</sup> mobilization, while the pERK signal was similar to that observed in Fig. 4B (A. Filby, B. Seddon, M. Veldkoe, J. M. Sanchez-Morgado, M. Smida, J. A. Lindquist, and R. Zamoyska, unpublished data).

Stimulation of WT F5 T cells revealed that at the highest peptide doses, up to 55% of cells had substantial upregulation of intracellular pERK at 15 min (Fig. 1A), and the proportions of cells responding titrated linearly with increasing doses of peptide (Fig. 1B). The specificity of the pERK Ab was controlled by pretreating samples with the highest dose of peptide with the MEK inhibitor, UO126, which reduced staining to background levels (Fig. 5A). The availability of Lck had a direct effect on the number of cells that were able to phosphorylate ERK, which was about 10 to 15% of that for WT cells, and this percentage was consistent across the antigen titration (Fig. 5A and B). However, lack of Lck had much less influence on the quantity of intracellular pERK per cell, as the MFI values were reduced by about 20% (Fig. 5C). Moreover, defects in ERK activity were shown to be restricted to signals downstream of the TCR, as stimulation with phorbol esters provoked equivalent responses in all samples (Fig. 5A). Interestingly, stimulation of Fyn<sup>-/-</sup> CD8 T cells showed dose response curves identical to those of WT cells in terms of the numbers of cells that were stimulated (Fig. 5A and B) but, on average, greater reductions in MFI (~40%) than those for

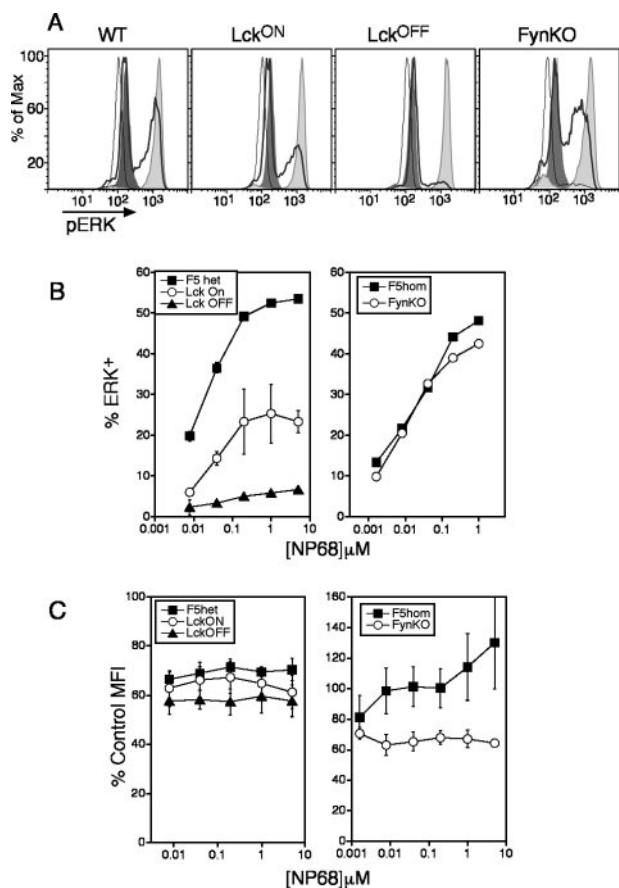


FIG. 5. Fyn contributes to the magnitude of ERK activation in F5 T cells in response to peptide stimulation. (A) FACS histograms showing intracellular pERK staining following stimulation for 15 min with medium (thin line), 1  $\mu$ M NP68 peptide (thick line), 1  $\mu$ M NP68 peptide plus UO126 MEK inhibitor (dark fill), or PDBu (light fill) for F5 T cells on the indicated backgrounds. (B) Dose-response curves for triplicate samples  $\pm$  standard errors, showing the percentages of ERK<sup>+</sup> cells after 15 min of stimulation with peptide. Data are representative of at least three independent experiments. (C) Mean MFIs  $\pm$  standard errors for ERK<sup>+</sup> cells in response to peptide titration from three to four independent experiments calculated from the formula  $(\text{exp} - b/g) / (\text{total} - b/g) \times 100\%$ , where exp is the MFI of the ERK<sup>+</sup> gate from the peptide-stimulated sample,  $b/g$  is the MFI of the UO126-inhibited sample, and total is the MFI of the ERK<sup>+</sup> gate from the PDBu-stimulated sample.

cells lacking Lck. Moreover, the durations of the ERK responses in all groups were equivalent and could be followed from 5 min to at least 4 h (data not shown). These data indicate that signals downstream of Fyn play a substantial role in the activation of the ERK/MAPK pathway in primary T cells by influencing the magnitude of the pERK signal, whereas signals downstream of Lck seem primarily to regulate the threshold of triggering.

**Lck and Fyn activate ERKs through different pathways.** Given the profound defect in PLC- $\gamma$ 1 activation in the Lck<sup>OFF</sup> cells, we asked which signaling pathways might be activating ERK downstream of Lck and Fyn. One effector of Ras in T cells is the guanine nucleotide exchange factor (GEF) RasGRP1, which is recruited to the plasma membrane upon T-cell stimulation. Signals through the TCR generally result in

PLC- $\gamma$ 1 activation, which converts phosphatidylinositol 4,5 bisphosphate (PIP<sub>2</sub>) into inositol 3,4,5 triphosphate (InsP<sub>3</sub>) and DAG, with the latter promoting membrane recruitment of RasGRP1 through its DAG binding C1 domain (12). In Lck<sup>-/-</sup> cells, there was poor phosphorylation of LAT Y136, the main residue which recruits PLC- $\gamma$ 1, concomitant with reduced phosphorylation of PLC- $\gamma$ 1 itself, suggesting that DAG production was likely to be compromised. To see whether this was true, we looked for DAG production in two ways, first, by looking for recruitment of RasGRP1 to the plasma membrane after activation, and second, by investigating the activation of PKD, which is also dependent on DAG (31).

To facilitate subcellular fractionation, which is difficult to achieve cleanly with resting naive peripheral T cells, CD4<sup>+</sup> T cell blasts were generated from WT and Lck<sup>OFF</sup> mice, allowed to rest, and then restimulated with anti-CD3 plus anti-CD4 in order to examine RasGRP1 translocation to the plasma membrane. Importantly, restimulation of CD4 T-cell blasts was comparable to that of naive CD4 T cells in that both had severely reduced ZAP-70/PLC- $\gamma$ 1 activation and both retained ERK activation (data not shown). Membrane fractions were prepared from stimulated cells, run on SDS-PAGE gels, and analyzed by Western blotting for the presence of RasGRP1. In WT cells, a significant fraction of RasGRP1 could be seen to translocate to the plasma membrane after stimulation with CD3 plus CD4, whereas in the absence of Lck, the RasGRP1 signal after stimulation was the same as that of the background, indicating a clear reduction in DAG production and RasGRP recruitment in these cells (Fig. 6A). Again, we could show that there were no global defects in RasGRP translocation in the absence of Lck, as recruitment to the membrane occurred in all samples after phorbol ester treatment. To confirm that DAG production was impaired in the absence of Lck in primary ex vivo T cells also, we examined the phosphorylation of PKD on S916, which has previously been shown to be dependent on DAG (31). Stimulation of naive CD4 T cells from either WT or Lck<sup>ON</sup> mice with CD3-plus-CD4 Ab resulted in the distinct appearance of a phosphorylated PKD species at 2 min, which was recognized by S916-specific antisera (Fig. 6B). In contrast, Lck<sup>OFF</sup> cells lacked this species, confirming that DAG production is compromised in cells lacking Lck. Together, these data suggest that the activation of ERKs in the absence of Lck is via a pathway which does not involve PLC- $\gamma$ 1/RasGRP1.

Production of DAG also results in the membrane localization and activation of PKC $\theta$ , which is required for the activation of mature T cells and the production of IL-2 (3). We found that in WT cells, CD3 and CD3-plus-CD4 stimulation resulted in robust phosphorylation of T538 on PKC $\theta$ , while cells from Lck<sup>OFF</sup> mice showed little to no PKC $\theta$  phosphorylation (Fig. 6C). Once again, in the presence of low levels of Lck (Lck<sup>ON</sup> T cells), CD4 coligation with the TCR during stimulation was required to reveal good phosphorylation of PKC $\theta$ , confirming that its activation is also dependent on the activity of Lck.

Finally, another TCR-associated molecule whose activation has been suggested to feed into the Ras MAPK pathway is the adapter molecule Shc. By means of its SH3 domain, Shc has been shown to bind Grb2-SOS and hence activate the Ras/

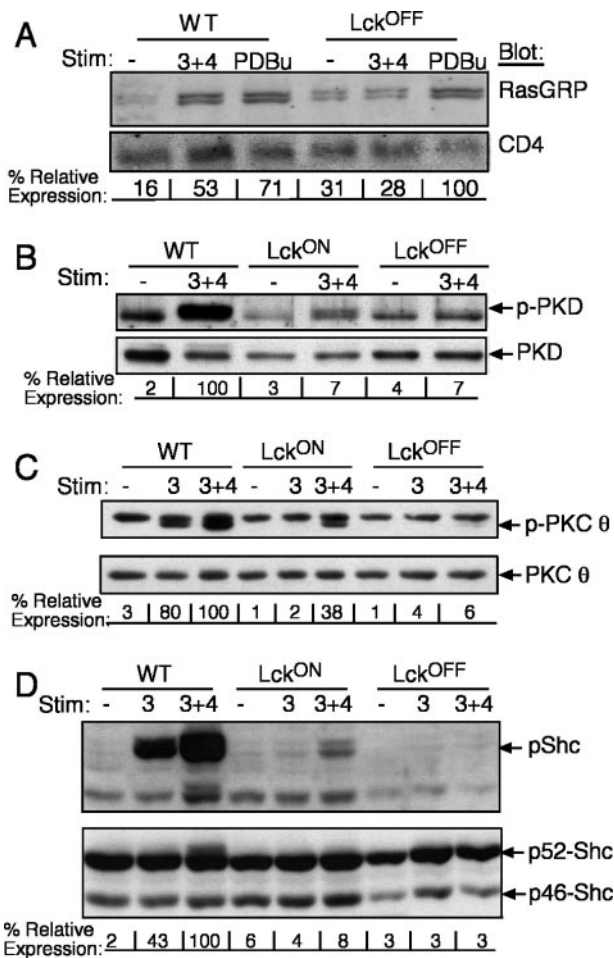


FIG. 6. Absence of Lck compromises recruitment of RasGRP, DAG production, and phosphorylation of PKC $\theta$  and Shc. CD4 T-cell blasts were stimulated with anti-CD3 plus anti-CD4 Abs or PDBu, as indicated, for 2 min at 37°C. (A) Cell membranes were prepared and probed for RasGRP and CD4, and relative RasGRP expression was quantified by infrared imaging using the Li-Cor Odyssey system. Total cell lysates from naïve, purified CD4 T cells were stimulated and probed for PKD pS916 (B), pPKC $\theta$  on T538 (C), and pShc Y239/240 (D) and visualized with chemiluminescence. Each blot is representative of two to four replicates, and percent relative expression was determined as for Fig. 2.

ERK pathway (58). However, we found that in the absence of Lck, there was a severe decrease in both the p46 and the p52 phosphorylated forms of Shc upon activation (Fig. 6D). Moreover, CD4 coengagement increased phosphorylation of Shc considerably, especially in the presence of low levels of Lck, confirming previous reports that Shc is a good substrate of Lck (15, 51, 58) and that Shc is unlikely to be contributing to activation of the ERK pathway in the absence of Lck.

**Lck is required for proliferation and cytokine production.** We examined the downstream consequences of receptor triggering for primary CD4<sup>+</sup> cells in the absence of Lck. We showed previously that CD69 upregulation occurs in the absence of Lck but that the proportion of cells stimulated and the extent of CD69 expression are both reduced (38). In total peripheral LN preparations, CD8 cells respond more vigorously than CD4 cells and dominate the culture; therefore, we

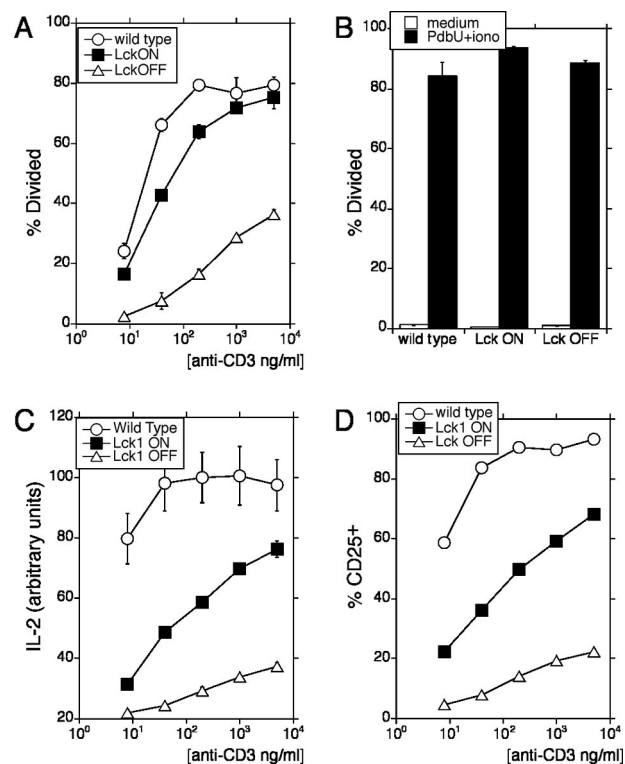


FIG. 7. Impaired T-cell proliferation and IL-2 secretion in the absence of Lck. Proliferation of CFSE-labeled, purified CD4<sup>+</sup> T cells stimulated with anti-CD3 plus anti-CD28 (A) or PDBu plus ionomycin (B) for 72 h. (C) IL-2 was measured from supernatant after 24 h culture, in response to anti-CD3 plus 1  $\mu$ g/ml anti-CD28, and (D) expression of CD25 was determined. Graphs show means  $\pm$  standard deviations for triplicate points.

examined the fate of purified CD4 cells after stimulation with Ab, although similar data have been obtained for CD8 T cells (Filby et al., unpublished data). We used the cell-tracking dye CFSE to monitor proliferation and assessed early activation events, such as CD25 expression and IL-2 production. Purified CFSE-labeled CD4<sup>+</sup> T cells were stimulated in vitro with soluble anti-CD3 plus anti-CD28. After 72 h, the percentage of cells in each CFSE peak was enumerated and used to calculate the percentage of the starting population which had entered division. WT and Lck<sup>ON</sup> cells behave remarkably similarly over a range of Ab concentrations (Fig. 7A), suggesting that despite some early deficiencies in Lck-mediated signaling, upregulation of Lck upon activation (Fig. 1) restored the capacity of these cells to respond. As previously reported (38, 47), Lck-deficient CD4 T cells responded poorly to anti-CD3 stimulation, suggesting that the threshold for triggering such cells into division is substantially altered. At higher concentrations of CD3 Ab, Lck<sup>-/-</sup> cells did enter division, and remarkably, for those cells that had crossed this threshold, the numbers of divisions that they underwent were close to those achieved by WT cells, with division indexes of 2.6 for WT, 2.8 for Lck<sup>ON</sup>, and 1.8 for Lck<sup>OFF</sup> cells. Treatment with PDBu in combination with ionomycin restored the ability of Lck<sup>-/-</sup> T cells to proliferate to a level comparable to those for both WT and Lck1<sup>ON</sup> CD4<sup>+</sup> T cells (Fig. 7B). This result indicated that there were no other intrinsic defects in Lck<sup>-/-</sup> cells that could



not be circumvented by bypassing TCR-dependent activation of DAG and  $\text{Ca}^{2+}$ .

Consistent with the defects in  $\text{Ca}^{2+}$ , PLC- $\gamma$ 1, and PKC $\theta$  activation, Lck-deficient cells were very poor in secreting IL-2 (Fig. 7C). In addition, upregulation of CD25 was compromised (Fig. 7D). These parameters were also defective in the cells which expressed low levels of Lck. At first sight, this seems to be contradictory to the proliferation data, which were the same between WT and Lck<sup>ON</sup> cells. However, proliferation was assessed at 72 h, by which time the expression of Lck was substantially upregulated (Fig. 1) in contrast to what was found for the CD25 and IL-2 measurements, which were carried out at 24 h. The upregulation of CD25 and the transcription of the IL-2 gene in the initial part of the response are driven directly by TCR signals, which are highly dependent on Lck expression. In contrast, maintenance of proliferation seems to be less dependent on Lck.

## DISCUSSION

Transgenic mice expressing Lck in an inducible fashion provide a novel opportunity to assess the role of Lck in the induction of T-cell responses in primary naïve T cells. By examining proximal biochemical signals transduced by ligation of the TCR either alone or in combination with CD4, we were able to discriminate pathways that were primarily induced by Lck from those that were correspondingly influenced by activation of both Lck and Fyn. From these data, we showed that Lck profoundly influences the threshold at which cells are triggered, and biochemically, this correlated with the ability of Lck to induce signaling pathways leading to PLC- $\gamma$ 1 activation, inositol phospholipid hydrolysis, and the mobilization of intracellular  $\text{Ca}^{2+}$ . In contrast, we found that both Lck and Fyn contributed to activation of the ERK/MAPK pathway and that they seem to access this pathway through distinct upstream mediators.

The influence of Lck on early signaling events, such as activation of ZAP-70, was evident by the absence of the phosphorylation of ZAP Y319 in Lck<sup>-/-</sup> cells and its reduced kinase activity, resulting in the decreased phosphorylation of LAT on several key residues. Phosphorylation of LAT Y136 is important for recruitment of PLC- $\gamma$ 1 (1, 28, 34, 42, 59), and diminished phosphorylation of this residue could largely explain the inability of Lck<sup>-/-</sup> cells to link to the phosphatidylinositol and PKC signaling pathways. In addition, mutation of LAT Y195 was shown to disrupt interaction with the adapter molecule GADS, which is responsible for the recruitment of Slp-76. We found that phosphorylation of Slp-76 was also severely defective in the absence of Lck (data not shown), suggesting that the GADS/Slp-76/PLC- $\gamma$ 1 complex would be unlikely to assemble efficiently, which could also contribute to the failure to activate PLC- $\gamma$ 1. Surprisingly, despite these defects in PLC- $\gamma$ 1 signaling, a proportion of Lck<sup>-/-</sup> cells were triggered to respond. By single-cell FACS analysis, these cells were shown to phosphorylate ERK at levels that were ~80% of those for WT cells. This ability to activate ERKs required expression of Fyn, and moreover, for WT primary T cells, we showed that Fyn contributes substantially to the magnitude of ERK phosphorylation.

There are two major GEFs for Ras in T cells, RasGRP1 and

SOS (16). The former is recruited to the membrane by the production of DAG and has been shown to be important for thymocyte differentiation and peripheral T-cell expansion (11, 35) and recently to be the major activator of ERKs in Jurkat T cells (37). However, it is unlikely that RasGRP1 was the major contributor to the activation of the Ras/ERK pathway in the absence of Lck, as there appeared to be insufficient DAG production to result in Ser phosphorylation of PKD or to recruit RasGRP1 to the plasma membrane efficiently. Moreover, activation of PKC $\theta$  was also severely compromised in the absence of Lck, which may have contributed to the failure to activate RasGRP1, as activation of novel PKCs has recently been shown to contribute to RasGRP1 signaling in Jurkat cells (37). It is likely, therefore, that the activation of ERK in the absence of Lck proceeded primarily via SOS, although this was unlikely to involve the adapter Shc, whose phosphorylation, as confirmed here, is extremely dependent on the activation of Lck (15). The most likely scenario in the absence of Lck is that Grb2/SOS is directly recruited to LAT and acts as the Ras GEF, which results in the partial phosphorylation of ERKs. Certainly, Fyn was able to promote limited kinase activity for ZAP-70 and some LAT Tyr phosphorylation, particularly at Y175 and Y235, residues known to bind Grb2. We were unable to demonstrate experimentally a direct association between LAT and Grb2/SOS in either primary WT or Lck-deficient cells, suggesting that such an association could be at low stoichiometry. It seems, therefore, that unlike in Jurkat cells, where ERK activation proceeds almost exclusively through RasGRP1 and cannot be compensated by recruitment of SOS (37), in primary T cells, both Src kinases contribute to ERK activity. The expression of Fyn has been reported to be very low in some Jurkat cell lines (9), in contrast to the high expression in primary T cells, which may well explain some of these differences.

The primary consequence of the lack of Lck in naïve T cells was to raise the activation threshold, resulting in a 100-fold shift in the dose-response curve. The percentage of cells that were triggered to divide corresponded to the proportion that were able to phosphorylate ERK and that showed an increased  $\text{Ca}^{2+}$  flux at later time points. It is possible that although the initial Ca flux is severely compromised in these cells because of the deficiency in PLC- $\gamma$ 1 activation and  $\text{InsP}_3$  production, there remains sufficient residual activity of these signaling pathways or even activation of other second messengers, such as cyclic ADP-ribose, which can result in the opening of  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  channels in the plasma membrane and thereby increase intracellular Ca (54). Certainly, the ability to induce IL-2 transcription is severely compromised in the absence of Lck, suggesting that NFAT activation is considerably reduced. Interestingly, if intracellular IL-2 is visualized following recall with a 4-h PDBu and ionomycin pulse, a significant proportion of the cells are found to produce IL-2 (data not shown). Therefore, the signals received in the absence of Lck are sufficient to open the locus but insufficient to induce normal levels of IL-2 transcription.

It is clear from these data that Lck and Fyn play distinct roles in primary T-cell activation even though some signaling pathways may be targeted by both kinases. One critical difference between Lck and Fyn is that, by virtue of their unique N-terminal domains, each is resident in a separate subcellular

compartment of the plasma membrane. Fyn is mainly associated with lipid rafts and the TCR, while Lck is associated with the coreceptors, and the active pool seems to be held outside lipid rafts (14, 56). Signaling through anti-CD3 alone is clearly less efficient than signaling through CD3 plus CD4, and the former is particularly compromised in naïve T cells that lack Fyn, even though their response to CD3-plus-CD4 coligation is relatively normal (46). In addition, coaggregation of TCR and CD4 can greatly increase basal Fyn activity (13). It has been suggested that one role of Fyn is to provide partial signals, such as those delivered by antagonist ligands (19), and our data would suggest that this may be through Fyn providing partial phosphorylation of LAT and thus separating the activation of the ERK pathway from the inositol phosphate/protein kinase C pathways. These data, together with the demonstration that Fyn and not Lck preferentially interacts with molecules that are negative regulators of T-cell activation, such as the PAG/Csk (29) and SAP/SLAM (25), suggest that the primary role of Fyn is to dampen T-cell responses, while Lck acts to amplify them.

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#### REFERENCES

- Aguado, E., S. Richelme, S. Nunez-Cruz, A. Miazek, A. M. Mura, M. Richelme, X. J. Guo, D. Sainy, H. T. He, B. Malissen, and M. Malissen. 2002. Induction of T helper type 2 immunity by a point mutation in the LAT adaptor. *Science* **296**:2036–2040.
- al-Ramadi, B. K., T. Nakamura, D. Leitenberg, and A. L. Bothwell. 1996. Deficient expression of p56(lck) in Th2 cells leads to partial TCR signaling and a dysregulation in lymphokine mRNA levels. *J. Immunol.* **157**:4751–4761.
- Altman, A., and M. Villalba. 2003. Protein kinase C-theta (PKCtheta): it's all about location, location, location. *Immunol. Rev.* **192**:53–63.
- Appleby, M. W., J. A. Gross, M. P. Cooke, S. D. Levin, X. Qian, and R. M. Perlmutter. 1992. Defective T cell receptor signaling in mice lacking the thymic isoform of p59fyn. *Cell* **70**:751–763.
- Brdickova, N., T. Brdicka, P. Angelisova, O. Horvath, J. Spicka, I. Hilgert, J. Paces, L. Simeoni, S. Kliche, C. Merten, B. Schraven, and V. Horejsi. 2003. LIME: a new membrane raft-associated adaptor protein involved in CD4 and CD8 coreceptor signaling. *J. Exp. Med.* **198**:1453–1462.
- Chan, A. C., M. Iwashima, C. W. Turck, and A. Weiss. 1992. ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell* **71**:649–662.
- Davidson, D., X. Shi, S. Zhang, H. Wang, M. Nemer, N. Ono, S. Ohno, Y. Yanagi, and A. Veillette. 2004. Genetic evidence linking SAP, the X-linked lymphoproliferative gene product, to Src-related kinase FynT in T(H)2 cytokine regulation. *Immunity* **21**:707–717.
- Denny, M. F., H. C. Kaufman, A. C. Chan, and D. B. Straus. 1999. The lck SH3 domain is required for activation of the mitogen-activated protein kinase pathway but not the initiation of T-cell antigen receptor signaling. *J. Biol. Chem.* **274**:5146–5152.
- Denny, M. F., B. Patai, and D. B. Straus. 2000. Differential T-cell antigen receptor signaling mediated by the Src family kinases Lck and Fyn. *Mol. Cell Biol.* **20**:1426–1435.
- Di Bartolo, V., D. Mege, V. Germain, M. Pelosi, E. Dufour, F. Michel, G. Magistrelli, A. Isacchi, and O. Acuto. 1999. Tyrosine 319, a newly identified phosphorylation site of ZAP-70, plays a critical role in T cell antigen receptor signaling. *J. Biol. Chem.* **274**:6285–6294.
- Dower, N. A., S. L. Stang, D. A. Bottorff, J. O. Ebinu, P. Dickie, H. L. Ostergaard, and J. C. Stone. 2000. RasGRP is essential for mouse thymocyte differentiation and TCR signaling. *Nat. Immunol.* **1**:317–321.
- Ebinu, J. O., D. A. Bottorff, E. Y. Chan, S. L. Stang, R. J. Dunn, and J. C. Stone. 1998. RasGRP, a Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. *Science* **280**:1082–1086.
- Filipp, D., B. L. Leung, J. Zhang, A. Veillette, and M. Julius. 2004. Enrichment of lck in lipid rafts regulates colocalized fyn activation and the initiation of proximal signals through TCR alpha beta. *J. Immunol.* **172**:4266–4274.
- Filipp, D., J. Zhang, B. L. Leung, A. Shaw, S. D. Levin, A. Veillette, and M. Julius. 2003. Regulation of Fyn through translocation of activated Lck into lipid rafts. *J. Exp. Med.* **197**:1221–1227.
- Fukushima, A., Y. Hatanaka, J. W. Chang, M. Takamatsu, N. Singh, and M. Iwashima. 2005. Lck couples Shc to TCR signaling. *Cell. Signal.* **18**:1182–1189.
- Genot, E., and D. A. Cantrell. 2000. Ras regulation and function in lymphocytes. *Curr. Opin. Immunol.* **12**:289–294.
- Hermiston, M. L., Z. Xu, R. Majeti, and A. Weiss. 2002. Reciprocal regulation of lymphocyte activation by tyrosine kinases and phosphatases. *J. Clin. Invest.* **109**:9–14.
- Hernandez-Hoyos, G., S. J. Sohn, E. V. Rothenberg, and J. Alberola-Ila. 2000. Lck activity controls CD4/CD8 T cell lineage commitment. *Immunity* **12**:313–322.
- Huang, J., K. Sugie, D. M. La Face, A. Altman, and H. M. Grey. 2000. TCR antagonist peptides induce formation of APC-T cell conjugates and activate a Rac signaling pathway. *Eur. J. Immunol.* **30**:50–58.
- Hur, E. M., M. Son, O. H. Lee, Y. B. Choi, C. Park, H. Lee, and Y. Yun. 2003. LIME, a novel transmembrane adaptor protein, associates with p56lck and mediates T cell activation. *J. Exp. Med.* **198**:1463–1473.
- Irvin, B. J., B. L. Williams, A. E. Nilson, H. O. Maynor, and R. T. Abraham. 2000. Pleiotropic contributions of phospholipase C- $\gamma$ 1 (PLC- $\gamma$ 1) to T-cell antigen receptor-mediated signaling: reconstitution studies of a PLC- $\gamma$ 1-deficient Jurkat T-cell line. *Mol. Cell Biol.* **20**:9149–9161.
- Karnitz, L., S. L. Sutor, T. Torigoe, J. C. Reed, M. P. Bell, D. J. McKean, P. J. Leibson, and R. T. Abraham. 1992. Effects of p56lck deficiency on the growth and cytolytic effector function of an interleukin-2-dependent cytotoxic T-cell line. *Mol. Cell Biol.* **12**:4521–4530.
- Krutzik, P. O., and G. P. Nolan. 2003. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. *Cytometry A* **55**:61–70.
- Latour, S., R. Roncagalli, R. Chen, M. Bakinowski, X. Shi, P. L. Schwartzberg, D. Davidson, and A. Veillette. 2003. Binding of SAP SH2 domain to FynT SH3 domain reveals a novel mechanism of receptor signaling in immune regulation. *Nat. Cell Biol.* **5**:149–154.
- Latour, S., and A. Veillette. 2003. Molecular and immunological basis of X-linked lymphoproliferative disease. *Immunol. Rev.* **192**:212–224.
- Legname, G., B. Seddon, M. Lovatt, P. Tomlinson, N. Sarner, M. Tolaini, K. Williams, T. Norton, D. Kioussis, and R. Zamoyska. 2000. Inducible expression of a p56Lck transgene reveals a central role for Lck in the differentiation of CD4 SP thymocytes. *Immunity* **12**:537–546.
- Leo, A., and B. Schraven. 2001. Adapters in lymphocyte signalling. *Curr. Opin. Immunol.* **13**:307–316.
- Lin, J., and A. Weiss. 2001. Identification of the minimal tyrosine residues required for linker for activation of T cell function. *J. Biol. Chem.* **276**:29588–29595.
- Lindquist, J. A., L. Simeoni, and B. Schraven. 2003. Transmembrane adapters: attractants for cytoplasmic effectors. *Immunol. Rev.* **191**:165–182.
- Lucas, J. A., A. T. Miller, L. O. Atherly, and L. J. Berg. 2003. The role of Tec family kinases in T cell development and function. *Immunol. Rev.* **191**:119–138.
- Matthews, S. A., E. Rozengurt, and D. Cantrell. 1999. Characterization of serine 916 as an in vivo autophosphorylation site for protein kinase D/protein kinase Cmu. *J. Biol. Chem.* **274**:26543–26549.
- Molina, T., K. Kishihara, D. Siderovski, W. van Ewijk, A. Narendran, E. Timms, A. Wakeham, C. Paige, K.-U. Hartmann, A. Veillette, D. Davidson, and T. Mak. 1992. Profound block in thymocyte development in mice lacking p56<sup>lck</sup>. *Nature* **357**:161–163.
- Olsozowy, M. W., P. L. Leuchtman, A. Veillette, and A. S. Shaw. 1995. Comparison of p56lck and p59fyn protein expression in thymocyte subsets, peripheral T cells, NK cells, and lymphoid cell lines. *J. Immunol.* **155**:4236–4240.
- Paz, P. E., S. Wang, H. Clarke, X. Lu, D. Stokoe, and A. Abo. 2001. Mapping the Zap-70 phosphorylation sites on LAT (linker for activation of T cells) required for recruitment and activation of signalling proteins in T cells. *Biochem. J.* **356**:461–471.
- Priatel, J. J., S. J. Teh, N. A. Dower, J. C. Stone, and H. S. Teh. 2002. RasGRP1 transduces low-grade TCR signals which are critical for T cell development, homeostasis, and differentiation. *Immunity* **17**:617–627.
- Reynolds, L. F., C. de Bettignies, T. Norton, A. Beeser, J. Chernoff, and V. L. Tybulewicz. 2004. Vav1 transduces T cell receptor signals to the activation of the Ras/ERK pathway via LAT, Sos, and RasGRP1. *J. Biol. Chem.* **279**:18239–18246.
- Roose, J. P., M. Mollenauer, V. A. Gupta, J. Stone, and A. Weiss. 2005. A diacylglycerol-protein kinase C-RasGRP1 pathway directs Ras activation upon antigen receptor stimulation of T cells. *Mol. Cell Biol.* **25**:4426–4441.
- Seddon, B., G. Legname, P. Tomlinson, and R. Zamoyska. 2000. Long-term survival but impaired homeostatic proliferation of naïve T cells in the absence of p56lck. *Science* **290**:127–131.
- Seddon, B., P. Tomlinson, and R. Zamoyska. 2003. Interleukin 7 and T cell receptor signals regulate homeostasis of CD4 memory cells. *Nat. Immunol.* **4**:680–686.
- Seddon, B., and R. Zamoyska. 2002. TCR signals mediated by Src family

- kinases are essential for the survival of naive T cells. *J. Immunol.* **169**:2997–3005.
41. **Sommers, C. L., R. K. Menon, A. Grinberg, W. Zhang, L. E. Samelson, and P. E. Love.** 2001. Knock-in mutation of the distal four tyrosines of linker for activation of T cells blocks murine T cell development. *J. Exp. Med.* **194**: 135–142.
  42. **Sommers, C. L., C. S. Park, J. Lee, C. Feng, C. L. Fuller, A. Grinberg, J. A. Hildebrand, E. Lacana, R. K. Menon, E. W. Shores, L. E. Samelson, and P. E. Love.** 2002. A LAT mutation that inhibits T cell development yet induces lymphoproliferation. *Science* **296**:2040–2043.
  43. **Stefanova, L. B., Hemmer, M. Vergelli, R. Martin, W. E. Biddison, and R. N. Germain.** 2003. TCR ligand discrimination is enforced by competing ERK positive and SHP-1 negative feedback pathways. *Nat. Immunol.* **4**:248–254.
  44. **Stein, P. L., H. M. Lee, S. Rich, and P. Soriano.** 1992. pp59fyn mutant mice display differential signaling in thymocytes and peripheral T cells. *Cell* **70**: 741–750.
  45. **Straus, D. B., and A. Weiss.** 1992. Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell* **70**:585–593.
  46. **Sugie, K., M. S. Jeon, and H. M. Grey.** 2004. Activation of naive CD4 T cells by anti-CD3 reveals an important role for Fyn in Lck-mediated signaling. *Proc. Natl. Acad. Sci. USA* **101**:14859–14864.
  47. **Trobridge, P. A., and S. D. Levin.** 2001. Lck plays a critical role in Ca(2+) mobilization and CD28 costimulation in mature primary T cells. *Eur. J. Immunol.* **31**:3567–3579.
  48. **Utting, O., S. J. Teh, and H. S. Teh.** 1998. T cells expressing receptors of different affinity for antigen ligands reveal a unique role for p59fyn in T cell development and optimal stimulation of T cells by antigen. *J. Immunol.* **160**:5410–5419.
  49. **van Oers, N. S., N. Killeen, and A. Weiss.** 1996. Lck regulates the tyrosine phosphorylation of the T cell receptor subunits and ZAP-70 in murine thymocytes. *J. Exp. Med.* **183**:1053–1062.
  50. **van Oers, N. S., N. Killeen, and A. Weiss.** 1994. ZAP-70 is constitutively associated with tyrosine-phosphorylated TCR zeta in murine thymocytes and lymph node T cells. *Immunity* **1**:675–685.
  51. **Walk, S. F., M. E. March, and K. S. Ravichandran.** 1998. Roles of Lck, Syk and ZAP-70 tyrosine kinases in TCR-mediated phosphorylation of the adapter protein Shc. *Eur. J. Immunol.* **28**:2265–2275.
  52. **Williams, B. L., B. J. Irvin, S. L. Sutor, C. C. Chini, E. Yacyshyn, J. Bubeck Wardenburg, M. Dalton, A. C. Chan, and R. T. Abraham.** 1999. Phosphorylation of Tyr319 in ZAP-70 is required for T-cell antigen receptor-dependent phospholipase C-gamma1 and Ras activation. *EMBO J.* **18**:1832–1844.
  53. **Winkler, D. G., I. Park, T. Kim, N. S. Payne, C. T. Walsh, J. L. Strominger, and J. Shin.** 1993. Phosphorylation of Ser-42 and Ser-59 in the N-terminal region of the tyrosine kinase p56lck. *Proc. Natl. Acad. Sci. USA* **90**:5176–5180.
  54. **Winslow, M. M., J. R. Neilson, and G. R. Crabtree.** 2003. Calcium signalling in lymphocytes. *Curr. Opin. Immunol.* **15**:299–307.
  55. **Yamashita, M., K. Hashimoto, M. Kimura, M. Kubo, T. Tada, and T. Nakayama.** 1998. Requirement for p56(lck) tyrosine kinase activation in Th subset differentiation. *Int. Immunol.* **10**:577–591.
  56. **Yasuda, K., M. Nagafuku, T. Shima, M. Okada, T. Yagi, T. Yamada, Y. Minaki, A. Kato, S. Tani-Ichi, T. Hamaoka, and A. Kosugi.** 2002. Cutting edge: fyn is essential for tyrosine phosphorylation of csk-binding protein/phosphoprotein associated with glycolipid-enriched microdomains in lipid rafts in resting T cells. *J. Immunol.* **169**:2813–2817.
  57. **Zamojska, R., A. Basson, A. Filby, G. Legname, M. Lovatt, and B. Seddon.** 2003. The influence of the src-family kinases, Lck and Fyn, on T cell differentiation, survival and activation. *Immunol. Rev.* **191**:107–118.
  58. **Zhang, L., U. Lorenz, and K. S. Ravichandran.** 2003. Role of Shc in T-cell development and function. *Immunol. Rev.* **191**:183–195.
  59. **Zhang, W., R. P. Tribble, M. Zhu, S. K. Liu, C. J. McGlade, and L. E. Samelson.** 2000. Association of Grb2, Gads, and phospholipase C-gamma 1 with phosphorylated LAT tyrosine residues. Effect of LAT tyrosine mutations on T cell antigen receptor-mediated signaling. *J. Biol. Chem.* **275**: 23355–23361.