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Superantigen Stimulation Reveals the Contribution of Lck to Negative Regulation of T Cell Activation

Gabriel Criado and Joaquin Madrenas

The conventional paradigm of T cell activation through the TCR states that Lck plays a critical activating role in this signaling process. However, the T cell response to bacterial superantigens does not require Lck. In this study we report that not only is Lck dispensable for T cell activation by superantigens, but it actively inhibits this signaling pathway. Disruption of Lck function, either by repression of Lck gene expression or by selective pharmacologic inhibitors of Lck, led to increased IL-2 production in response to superantigen stimulation. This negative regulatory effect of Lck on superantigen-induced T cell responses required the kinase activity of Lck and correlated with early TCR signaling, but was independent of immunological synapse formation and TCR internalization. Our data demonstrate that the multistage role of Lck in T cell signaling includes the activation of a negative regulatory pathway of T cell activation. The Journal of Immunology, 2004, 172: 222–230.

Engagement of the Ag receptor on T lymphocytes triggers a complex signaling network that culminates in the activation and differentiation of these cells. One of the earliest detectable events in this process is the phosphorylation of tyrosine residues in motifs within the TCR-associated CD3 chains by the Src-related tyrosine kinase Lck (1). Tyrosine phosphorylation of these motifs leads to the recruitment and activation of the ZAP-70 kinase and phosphorylation of the linker for activated T cells (LAT), which then provides multiple docking sites for the assembly of signalosomes that activate downstream signaling pathways (2). The importance of Lck in this activation process is highlighted by the abnormalities in cells and animals lacking Lck. For example, the IcaM1.6 line, a Lck-deficient variant of the Jurkat T cell line, does not respond to TCR ligation by Abs, and this defect is reversed upon transfection with full-length Lck cDNA (3). More dramatically, Lck knockout mice have arrested thymocyte development at the double-negative stage that translates into a significant loss of peripheral T cells (4). Similarly, patients with deficient Lck activity show CD4+ T lymphopenia (5, 6).

In contrast to the above-mentioned evidence, several observations suggest that Lck may play a negative regulatory role in TCR-dependent signal transduction. First, Lck is involved in ligand-induced TCR internalization and thus may regulate the availability of TCR and the magnitude of T cell signaling (7–11). Second, separate ligation of TCR and CD4 causes Lck activation and inhibits T cell responses by priming CD4+ T cells for death (12, 13). And third, CD4-Lck fusion molecules without kinase activity enhance T cell responses (14, 15). Together, these findings are consistent with the existence of an Lck-mediated negative feedback on T cell activation.

More direct evidence for Lck as a negative regulator of T cell activation is provided by three separate observations. The first is that Lck activity increases after TCR engagement with some antagonist ligands (16). The second observation is that Th2 T cell lines rendered Lck deficient by transfection of Lck antisense DNA show enhanced cytokine production (17). The third observation is that peripheral T cells from patients with deficient Lck activity show hyper-responsiveness to allostimulation (6). These observations prompted us to hypothesize the existence of a Lck-dependent negative feedback pathway in T cell activation.

To test this hypothesis, it was first necessary to identify an experimental system in which Lck is not essential for TCR-dependent T cell activation. Such a system would allow us to determine the degree to which Lck contributes to down-regulation of T cell activation. Among the conditions of T cell activation that can occur in the absence of functional Lck (4, 6, 18–20), we chose T cell stimulation with bacterial superantigens (SAg) and APCs. After this type of stimulation, biochemical (e.g., extracellular signal-regulated kinase (ERK) activation) and functional (e.g., IL-2 production) responses are observed in Lck-deficient T cells; thus, it serves as a model of Lck-independent T cell activation. Given the value of Jurkat T cells to characterize the events involved in early TCR-mediated signaling and the availability of genetic mutants of these cells, we measured the response of wild-type Jurkat T cells, Lck-deficient Jurkat cells (JCaM1.6), and several Lck-reconstituted Jurkat variants to bacterial SAg. Using this system, we provide for the first time genetic, pharmacological, and functional evidence of the contribution of Lck to the down-regulation of T cell activation and cytokine production.

Materials and Methods

Cells

Human PBMC were isolated from healthy volunteers by Ficoll gradient centrifugation (Ficoll-Paque Plus; Amersham Pharmacia Biotech, Piscataway, NJ). Peripheral blood T cells were isolated from PBMC by negative selection with a human T cell enrichment cocktail (StemCell Technologies, Vancouver, Canada). Mitogen-activated T cells and monocyte-derived dendritic cells (MD-DC) were generated from PBMC that were allowed to adhere to six-well plates for 2 h at 37°C. Nonadherent cells enriched in

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lymphocytes were collected and treated with PMA (1 ng/ml) and ionomycin (100 ng/ml; Sigma-Aldrich Canada, Oakville, Canada). After 72 h, cells were washed four times with fresh medium and allowed to rest for at least 24 h. For MD-DC generation, adherent cells were treated with GM-CSF (1000 U/ml) and IL-4 (1000 U/ml; R&D Systems, Minneapolis, MN) as previously described (21, 22) for 4–6 days.

E6.1 cells, Lck-deficient JCaM1.6 cells, and ZAP-70-deficient P116 cells were purchased from American Type Culture Collection (Manassas, VA). JCaM1.6 cells expressing kinase-dead (K273R) Jck (JcMLck KD) cells (23), JCI/Lck cells (24), LAT-deficient JcaM2.5 cells, and LAT-reconstituted JcaM2.5 B3 cells (25) were provided by Dr. R. Wange (National Institutes of Health, Baltimore, MD). The lymphoblastoid B cell line LG2 was provided by Dr. E. Long (National Institutes of Health, Bethesda, MD). The lymphoblastoid B cell line .45 retrovirally transfected with gfp was a gift from Dr. B. Carreno (Wyeth Research, Cambridge, MA). All cell lines were maintained in RPMI 1640/10% FCS and standard supplements.

Abs and reagents

The following Abs were used for biochemical experiments: anti-active ERK mAb E10 (Cell Signaling Technology, Beverly, MA), anti-ERK rabbit polyclonal antiserum (StressGen, Victoria, Canada), antiphosphotyrosine mAb 4G10 (provided by Dr. B. Dukker, Oregon Health Sciences University, Portland, OR), anti-LAT and anti-Lck rabbit polyclonal Abs (Upstate Biotechnology, Lake Placid, NY), anti-ZAP-70 rabbit polyclonal Ab (265), provided by Dr. J. M. Rojo (Centro de Investigaciones Biológicas, Madrid, Spain), goat anti-rabbit Ig HRP conjugated (Bio-Rad, Hercules, CA), and sheep anti-mouse Ig HRP-conjugated (Amersham Pharmacia Biotech). The following Abs were used for flow cytometry and confocal microscopy: FITC-, PE-, and PE-Cy5-labeled anti-CD3; PE-labeled anti-CD69; PE-labeled anti-CD25; and PE-labeled anti-LFA-1 (Bio-Rad, Hercules, CA), and sheep anti-mouse Ig HRP-conjugated (Amersham Pharmacia Biotech). The following Abs were used for flow cytometry and confocal microscopy: FITC-, PE-, and PE-Cy5-labeled anti-CD3; PE-labeled anti-CD69; PE-labeled anti-CD25; and PE-labeled anti-LFA-1 (Bio-Rad, Hercules, CA), and sheep anti-mouse Ig HRP-conjugated (Amersham Pharmacia Biotech). The following Abs were used for flow cytometry and confocal microscopy: FITC-, PE-, and PE-Cy5-labeled anti-CD3; PE-labeled anti-CD69; PE-labeled anti-CD25; and PE-labeled anti-LFA-1 (Bio-Rad, Hercules, CA), and sheep anti-mouse Ig HRP-conjugated (Amersham Pharmacia Biotech). The following Abs were used for flow cytometry and confocal microscopy: FITC-, PE-, and PE-Cy5-labeled anti-CD3; PE-labeled anti-CD69; PE-labeled anti-CD25; and PE-labeled anti-LFA-1 (Bio-Rad, Hercules, CA), and sheep anti-mouse Ig HRP-conjugated (Amersham Pharmacia Biotech). The following Abs were used for flow cytometry and confocal microscopy: FITC-, PE-, and PE-Cy5-labeled anti-CD3; PE-labeled anti-CD69; PE-labeled anti-CD25; and PE-labeled anti-LFA-1 (Bio-Rad, Hercules, CA), and sheep anti-mouse Ig HRP-conjugated (Amersham Pharmacia Biotech). The following Abs were used for flow cytometry and confocal microscopy: FITC-, PE-, and PE-Cy5-labeled anti-CD3; PE-labeled anti-CD69; PE-labeled anti-CD25; and PE-labeled anti-LFA-1 (Bio-Rad, Hercules, CA), and sheep anti-mouse Ig HRP-conjugated (Amersham Pharmacia Biotech). The following Abs were used for flow cytometry and confocal microscopy: FITC-, PE-, and PE-Cy5-labeled anti-CD3; PE-labeled anti-CD69; PE-labeled anti-CD25; and PE-labeled anti-LFA-1 (Bio-Rad, Hercules, CA), and sheep anti-mouse Ig HRP-conjugated (Amersham Pharmacia Biotech). The following Abs were used for flow cytometry and confocal microscopy: FITC-, PE-, and PE-Cy5-labeled anti-CD3; PE-labeled anti-CD69; PE-labeled anti-CD25; and PE-labeled anti-LFA-1 (Bio-Rad, Hercules, CA), and sheep anti-mouse Ig HRP-conjugated (Amersham Pharmacia Biotech). The following Abs were used for flow cytometry and confocal microscopy: FITC-, PE-, and PE-Cy5-labeled anti-CD3; PE-labeled anti-CD69; PE-labeled anti-CD25; and PE-labeled anti-LFA-1 (Bio-Rad, Hercules, CA), and sheep anti-mouse Ig HRP-conjugated (Amersham Pharmacia Biotech). The following Abs were used for flow cytometry and confocal microscopy: FITC-, PE-, and PE-Cy5-labeled anti-CD3; PE-labeled anti-CD69; PE-labeled anti-CD25; and PE-labeled anti-LFA-1 (Bio-Rad, Hercules, CA), and sheep anti-mouse Ig HRP-conjugated (Amersham Pharmacia Biotech). The following Abs were used for flow cytometry and confocal microscopy: FITC-, PE-, and PE-Cy5-labeled anti-CD3; PE-labeled anti-CD69; PE-labeled anti-CD25; and PE-labeled anti-LFA-1 (Bio-Rad, Hercules, CA), and sheep anti-mouse Ig HRP-conjugated (Amersham Pharmacia Biotech).
To test the effect of Lck expression on Jurkat T cell activation by staphylococcal SAg, we analyzed the SEE-induced response of Lck-deficient T cells stably reconstituted with full-length Lck cDNA under a Tet-repressible promoter (hereafter called JC1/Lck cells) (24). When JC1/Lck cells were stimulated with SEE in the presence of 10 ng/ml doxycycline, conditions that correlate with almost complete inhibition of Lck expression, we observed a significant enhancement of IL-2 production for each concentration of SEE (Fig. 2A). The enhancing effect on IL-2 production in response to SEE was inversely proportional to the amount of Lck present in the cell, and maximal enhancement of IL-2 production was detected at concentrations of doxycycline that repressed Lck expression below detection levels (Fig. 2B). Doxycycline did not affect IL-2 production when added to nontransfected JCaM1.6 cells. In addition, the increased IL-2 production observed in Lck-repressed cells was not due to less activation-induced cell death, as the level of activation-induced cell death, as determined by propidium iodide incorporation, was comparable in Lck-replete and Lck-deficient T cells (data not shown). These data demonstrate that repression of Lck expression enhances T cell responsiveness to SEE, and thus illustrate for the first time that in addition to its conventional activating role, Lck has a negative regulatory function on T cell activation in response to TCR engagement by SAg.

Next, we tested whether the inhibitory role of Lck on SEE-induced activation of Jurkat T cells required its kinase activity. If

![FIGURE 1. Src kinase inhibition differentially affects SAg-induced activation of freshly isolated T cells or mitogen primed human T cell blasts. A, PBMC were stimulated with SEE (1 ng/ml) or anti-CD3 OKT3 Ab (1 ng/ml) in the presence of different concentrations of the Src kinase inhibitor PP2 or the control compound PP3. B, Peripheral blood T cells and T cell blasts were stimulated with autologous MD-DC (ratio, 20 T cells/1 DC) and SEE (10 ng/ml) in the presence of the indicated amounts of PP2. After 16–24 h of culture, supernatants were collected and assayed for IL-2 content by ELISA. Results are representative of two separate experiments for each cell condition.](http://www.jimmunol.org/)

![FIGURE 2. Lck plays a negative regulatory role in SEE-induced T cell activation in Jurkat T cells. A, Lck-deficient T cells (JCaM1.6; ○ and ●) and doxycycline-repressible Lck-reconstituted T cells (JC1/Lck; ■ and □) were cultured overnight without (○ and ■) or with doxycycline (10 ng/ml; ● and □) and subsequently stimulated with APC and different concentrations of SEE. B, Jurkat JC1/Lck cells were cultured overnight without or with the indicated concentrations of doxycycline and stimulated with APCs and SEE (100 ng/ml) in the presence of the indicated concentrations of doxycycline. After 24 h, culture supernatants were collected and assayed for IL-2 content. B, Doxycycline-treated JC1/Lck cells were lysed, and Lck levels were assayed by immunoblot (bottom panel). The image was acquired in a Fluorchem 8800 station (Alpha Innotech, San Leandro, CA) and quantitated with Phoretix 1D software (Non Linear, Durham, NC).](http://www.jimmunol.org/)
so, this might explain the increased Lck kinase activity in response to TCR antagonists (16) and the enhanced responses of T cells expressing a kinase-dead CD4-Lck fusion protein (14, 15). We examined the effect of pharmacological inhibition of the Src kinases with PP2 on the response of Jurkat T cells to SEE and APC. In this cell line, in which Lck is the predominant Src kinase (24, 34), we found that pretreatment with PP2 enhanced the production of IL-2 in response to SEE and APC in a dose-dependent manner. Such an enhancing effect was specific to PP2 and was not detected with PP3 (Fig. 3A). Furthermore, the effect of PP2 on IL-2 production was detected when cells were pretreated or treated shortly after the initiation of TCR stimulation, but PP2 lost most of its enhancing potency when added after 1 h of stimulation (Fig. 3B). Analysis of IL-2 production upon titration of SEE stimulation showed that the enhancing effect of PP2 on T cell activation was functional along the full range of SEE concentrations tested (Fig. 3C, left panel). In sharp contrast, PP2 pretreatment completely inhibited the response of Lck-deficient T cells to the same type of stimulation, corroborating that the enhancing effect of PP2 required the Lck kinase activity (Fig. 3C, right panel). It is important to note that these results are not unique to SEE, as the same results were obtained when T cells were stimulated with the streptococcal SAg streptococcal mitogenic exotoxin-Z (data not shown). However, this effect was not seen with Jurkat T cells stimulated with

FIGURE 3. Pharmacological inhibition of Lck enhances SEE-induced T cell activation in Jurkat T cells. A, Jurkat E6.1 cells were pretreated for 30 min with different concentrations of PP2 or PP3 and stimulated with APCs and SEE (10 ng/ml). B, Jurkat E6.1 cells were left untreated, pretreated for 30 min with PP2 (20 μM), or treated at different times during the stimulation with APCs and SEE (10 ng/ml). C, Jurkat E6.1 and Lck-deficient JcaM1.6 cells were cultured for 30 min without or with the Src kinase inhibitor PP2 (20 μM) and stimulated with APCs and different amounts of SEE. Supernatants were collected after 24 h of culture and were assayed for IL-2 by ELISA. The inhibitor was maintained in the culture for the entire stimulation period in all experimental groups.

FIGURE 4. The inhibitory effect of Lck on T cell activation requires its kinase activity. A, Jurkat E6.1 cells, Lck-deficient JcaM1.6 cells, and JcaM1.6 cells stably transfected with wild-type Lck (JC1/Lck) or with a kinase-dead Lck (JLckKD) were cultured for 30 min without or with PP2 (20 μM) and stimulated with APCs and SEE (10 ng/ml). B, JC1/Lck cells were left untreated or were treated overnight with doxycycline (1000 ng/ml) to repress Lck expression or for 30 min with PP2 (20 μM) or with a combination of doxycycline and PP2, and stimulated with APCs and SEE (10 ng/ml). After 24 h, culture supernatants were collected and assayed for IL-2. A, The level of IL-2 production for each cell line was normalized to that reached in the absence of the inhibitor to account for interclonal variations. When used, PP2 and doxycycline were maintained in the culture during the stimulation.
bead-immobilized anti-CD3 Abs and soluble anti-CD28 Abs, conditions under which PP2 inhibited IL-2 production in both Lck-replete and Lck-deficient T cells (data not shown). Together, these results show that pharmacological inhibition of Lck activity enhances IL-2 production by Jurkat T cells in response to bacterial SAg and imply that the inhibitory signals delivered by Lck upon SEE stimulation are mediated by its kinase activity.

To further demonstrate that the negative regulatory function of Lck on SAg-induced T cell activation requires the kinase activity of Lck, we undertook a genetic approach. We compared the IL-2 response to SEE and APC of multiple Lck-deficient T cell clones stably reconstituted with functional Lck to that of T cell clones reconstituted with a kinase-dead Lck. We observed that the enhancing effect of PP2 on IL-2 production in response to SEE was detected in Lck-deficient T cells reconstituted with functional Lck (JC1/Lck), but not in Lck-deficient T cells reconstituted with a kinase-dead Lck (JLckKD; Fig. 4A). Thus, the kinase activity of Lck is critical for the negative regulatory pathway that down-regulates T cell activation in response to SEE. This was also demonstrated in the JC1/Lck cell line by comparing the effects of PP2 under conditions of Lck expression and Lck deficiency. In these cells we observed that when Lck is expressed, i.e., without doxycycline in the culture medium, PP2 had an enhancing effect on IL-2 production in response to SEE and APC (Fig. 4B). However, when the same T cells did not express Lck, i.e., with doxycycline in the medium, PP2 inhibited IL-2 production in response to SEE.

**FIGURE 5.** T cell-APC conjugation and immunological synapse formation in the absence of functional Lck. A, HE-loaded Jurkat E6.1 and JCaM1.6 cells (10⁶ cells/sample) were stimulated for 30 min with .45-gfp APC (10⁶ cells/sample) in the absence or the presence of SEE (100 ng/ml) and analyzed by flow cytometry. APCs were detected in the FL1 channel, T cells in the FL2 channel and APC-T cell conjugates were detected as dual-color events. A representative experiment is shown, indicating the percentage of conjugates for each condition. B, Data from four experiments were collected and analyzed for statistical significance by ANOVA (p < 0.0001) and Student’s t test (***, p < 0.01). Similar results were obtained when CFSE-loaded T cells and HE-loaded LG2 cells were assayed. C) Jurkat E6.1 and JCaM1.6 cells (10⁶ cells/sample) were stimulated for 30 min with .45-gfp cells (10⁵ cells/sample; signal in green) in the absence or the presence of SEE (100 ng/ml), stained with anti-CD3e-PE-Cy5 (blue signal) and anti-LFA-1-PE (red signal), and analyzed by confocal microscopy. D, Jurkat E6.1 (10⁶ cells/sample), untreated or treated with PP2 (20 μM), were stimulated for 30 min with LG2 cells in the presence of 100 ng/ml SEE, stained with anti-CD3e-PE-Cy5 (blue signal) and anti-LFA-1-PE (red signal), and analyzed by confocal microscopy. Note that for D, the LG2 APC were not labeled. White arrowheads point toward molecular clusters at the T cell:APC interface.
and APC (Fig. 4B). This result conclusively demonstrates that it is the kinase activity of Lck that plays a negative regulatory role on SAg-induced T cell activation. It also implies that the inhibition of IL-2 production by PP2 in T cells that do not express Lck is due to inhibition of Fyn, which then is essential for activation of these cells (19).

Next, we examined whether the negative regulatory role of Lck in the response to SEE was associated with changes in the ability to form T cell:APC conjugates and immunological synapses. After 30 min, we observed that the basal number of T:APC conjugates was lower in Lck-deficient T cells than in parental T cells (Fig. 5A). However, SEE stimulation consistently induced a 3-fold increase in the number of conjugates in both parental T cells and Lck-deficient JCaM1.6 T cells ($p < 0.01$; four experiments; Fig. 5B). This finding contrasts with the lack of conjugate formation in Lck-deficient T cells reported by Morgan et al. (29). However, it should be noted that these investigators examined early time points (10–15 min), whereas we studied later time points (30 min). Prolonged stimulation may allow the formation of stable conjugates and subsequent mature immunological synapses between T cells and APCs (35, 36). This claim is corroborated by the observation that the lack of Lck or the inhibition of Lck activity by PP2 did not prevent the formation of putative immunological synapses defined by the colocalization of TCR/CD3 and LFA-1 at a flat interface between T cell and APC (Fig. 5, C and D). Therefore, the negative role of Lck does not involve a gross alteration of the T cell:APC interaction.

Ligand-induced internalization and degradation of TCR can down-regulate T cell responses by decreasing the number of available TCR, thus reducing the magnitude of TCR-dependent signaling (8, 11, 37–39). This type of TCR internalization is dependent on Src kinases (7, 9, 10, 39, 40). One could argue that the enhancement of T cell activation in the absence of Lck activity could result from more TCR complexes available at the cell surface due to a decreased rate of TCR/CD3 internalization. However, this is not the case, because we found that TCR/CD3 internalization in the early stages of T cell activation (at 30 min, when inhibition of Lck activity had an enhancing effect) was comparable between Lck-deficient T cells and parental T cells (Fig. 6A). This result was confirmed in PP2-treated T cells (Fig. 6B) and in Lck-repressed T cells (data not shown) and was apparent at two different concentrations of SEE, confirming previous reports of SEE-induced TCR internalization in the absence of Lck (11, 41). These data show that the Lck-dependent negative feedback on SEE-induced T cell activation is not due to differences in the availability of the TCR. Interestingly, at later times of stimulation (15 h), we consistently observed increased CD3 expression in T cells lacking functional Lck to levels that were only seen in Lck-expressing T cells at higher concentrations of SEE, suggesting a potential effect of Lck on TCR re-expression.

The previous set of experiments demonstrated that membrane-associated events in response to bacterial SAg (e.g., T cell-APC conjugation, immunological synapse formation, TCR internalization) are comparable between Lck-expressing and Lck-deficient T cells and pointed to an intracellular signaling event(s) as the target(s) of Lck-mediated inhibition of T cell activation. Therefore, we examined the key early TCR signaling steps upon T cell stimulation by SEE under inhibition of Lck by PP2 (Fig. 7). As expected, SEE stimulation of Lck-expressing Jurkat T cells induced early tyrosine phosphorylation of TCR-$\xi$ and association with phospho-ZAP-70. In contrast, SEE stimulation in the presence of Lck inhibition induced tyrosine phosphorylation of TCR-$\xi$ with delayed kinetics, and under these conditions, ZAP-70 associated

**FIGURE 6.** SEE-induced TCR internalization in the absence of functional Lck. A, Jurkat E6.1 and JCaM1.6 cells (10⁶ cells/sample) were stimulated for 30 min with .45gfp cells (10⁶ cells/sample) in the absence or the presence of SEE (100 ng/ml). B, Jurkat E6.1 (10⁶ cells/sample), untreated or treated with PP2 (20 $\mu$M), were stimulated for different times with LG2 cells (10⁶ cells/sample) in the absence or the presence of different SEE concentrations. After stimulation, cells were stained with anti-CD3-PE-labeled (A) or anti-CD3-FITC-labeled (B) Abs and analyzed by flow cytometry. Data from three independent experiments were collected for each experimental condition, and the mean ± SD is shown. Similar results were obtained when staining with an anti-TCR Ab. LG2 and .45gfp cells are both APC expressing similar levels of HLA-DR1 and costimulatory molecules.

**FIGURE 7.** Tyrosine phosphorylation and association of TCR-$\xi$ and ZAP-70: effect of Lck inhibition. Parental Jurkat cells (E6.1) were stimulated with APCs and SEE (10 ng/ml) in the presence or the absence of PP2 (20 $\mu$M) for the indicated times. ZAP-70 immunoprecipitates from these cells were prepared and sequentially blotted for phosphotyrosine and ZAP-70. An APC sample containing the same number of APCs used to stimulate the T cells was analyzed (APCs), and a sample containing the eluate of the beads incubated with the Ab and lysis buffer in the absence of cellular extracts (Beads) was included.
with phospho-ζ, but was not phosphorylated, consistent with the lack of Lck activation.

Next, we analyzed the tyrosine phosphorylation of LAT, a cornerstone event for the assembly of signalosomes connecting the TCR-proximal events to effector signaling pathways (2, 25), and the kinetics of ERK activation, which may reflect distinct signaling patterns from the TCR (42). Remarkably, T cell stimulation with SEE induced LAT phosphorylation only in E6.1 cells (detectable after 1 min of stimulation and maximal after 15 min of stimulation), not in the Lck-deficient counterparts (Fig. 8A). Comprehensive dose-response and time-course analyses did not reveal any tyrosine phosphorylation of LAT in response to SEE in Lck-deficient T cells (Fig. 8A). This observation is remarkable because it occurred under conditions that induced maximal levels of IL-2 production by Lck-deficient T cells (Fig. 2). Activation of T cells by SEE in the absence of LAT phosphorylation confirms our previous findings of the ability of certain TCR stimuli to induce signal transduction leading to ERK activation and CD69 up-regulation in a LAT-independent manner (20, 28).

Analysis of the kinetics of ERK activation in response to SEE: APC revealed that Lck deficiency did not prevent ERK activation (Fig. 8B). Although Lck-deficient T cells required higher concentrations of SEE to activate ERK1/2 to levels similar to those seen in Lck-expressing T cells (Fig. 8B, left panel), the temporal profiles of ERK activation in response to SEE:APC were comparable in Lck-expressing T cells and Lck-deficient T cells (Fig. 8B, right panel). The observation of ERK activation in the absence of Lck activity and tyrosine phosphorylation of LAT in Jurkat cells is reminiscent of the phenotype of mice homozygous for a LAT mutation in which tyrosine 316 is replaced by phenylalanine (43, 44). These mice develop a lymphoproliferative disorder, and their T lymphocytes show intact ERK activation despite impaired phospholipase Cγ-1 activation.

The ability of Jurkat cells to respond to SEE in the absence of LAT was assessed by analyzing the up-regulation of the activation marker CD69 in the LAT-deficient variant JCaM2.5 cell line and the LAT-reconstituted counterpart JCaM2.5B3. Upon stimulation with SEE, LAT-deficient Jurkat cells were able to up-regulate the expression of CD69, although to a lesser extent than LAT-reconstituted JCaM2.5 cells (Fig. 9). As expected, SEE stimulation induced CD69 up-regulation in the parental E6.1 Jurkat cell line and the ZAP-70 deficient Jurkat cell line (P116), as we have previously reported (20). These results were further corroborated when CD25 up-regulation was analyzed in the same cell lines (data not shown). These data demonstrate that the Jurkat T cell response to SEE does not require ZAP-70 or LAT.

The specific biochemical pathway that mediates the negative regulatory function of Lck in vivo is currently under investigation. It may involve several mediators that can act as in vitro substrates

**FIGURE 8.** LAT phosphorylation-independent activation of ERK1/2 in Lck-deficient Jurkat T cells responding to SEE. A, Parental Jurkat cells (E6.1) and their Lck-deficient counterparts (JCaM1.6) were stimulated with APCs and different concentrations of SEE for 2 min (left panel) or with APCs and 10 ng/ml SEE for the indicated times (right panel). Lysates were immunoprecipitated with anti-LAT Ab and sequentially blotted for phosphotyrosine and LAT. B, Jurkat E6.1 and JCaM1.6 cells were stimulated with APCs and SEE for 2 min at the indicated concentrations (left panel) or with APCs and 10 ng/ml SEE for the indicated times (right panel). Whole cell lysates were prepared and sequentially immunoblotted for active ERKs and total ERK1/2.

**FIGURE 9.** ZAP-70-independent and LAT-independent T cell activation in response to SEE. Parental (E6.1), ZAP-70-deficient (P116), LAT-deficient (JCaM2.5), and LAT-reconstituted (JCaM2.5B3) Jurkat cells were stimulated with APCs in the presence or the absence of SEE (100 ng/ml) for 15 h. Cells were collected and analyzed for CD69 expression by flow cytometry.
of Lck. An obvious candidate for this role is Csk, a kinase that down-regulates TCR signaling upon relocation to lipid rafts in a Lck-dependent fashion (45–47). However, we have failed to notice a significant difference in the amount of Csk in lipid rafts of parental or Lck-deficient T cells upon stimulation with SEE (G. Criado and J. Madrenas, unpublished observations). Another potential mechanism to account for the negative role of Lck described in this study may be the recently reported Src homology protein tyrosine phosphatase-1-mediated negative feedback on T cell activation (48). Such an effect is based on the regulation of tyrosine-phosphorylated Src homology protein tyrosine phosphatase-1 binding to Lck by activated ERK. However, to be active, this mechanism requires early Lck-mediated tyrosine phosphorylation of SHP-1 combined with delayed and transient activation of ERK. These features of ERK activation are not those reported in this study to be associated with the negative role of Lck, i.e., early and sustained ERK activation (Fig. 7).

Together, our data provide for the first time functional evidence for the contribution of Lck to the negative regulation of T cell activation by SAg and offer biochemical evidence to explain the presence of some T cell responses in patients with defective Lck activity. The pathway involved in this novel role of Lck does not seem to be operational in T cell activation after TCR ligation with mAbs against TCR/CD3. It is not known whether it is operational in T cell activation after stimulation with peptide:MHC complexes. We have previously reported that persistent stimulation of T cells with Ag and APC correlates with a loss of TCR signaling and late inactivation of Lck (8), suggesting that the negative regulatory role of Lck may be active under these conditions of stimulation. The capacity of some SAg to dimerize may translate into oligomerization of the TCR to a degree similar to that seen with very potent Ag-induced stimulation, but in a unique way that facilitates the involvement of Fyn and plays down the need for Lck for the initiation of TCR signaling (49).

The negative regulation of SEE-induced T cell activation by Lck is probably coordinated with the other roles of Lck, i.e., initiation of signaling from nascent immunological synapses, stabilization of T:APC conjugates through integrin affinity maturation, and contribution to signaling from mature synapses (29, 50–53). It is tempting to speculate that the two pools of Lck detected at the immunological synapse recently reported by Ehrlich et al. (50) illustrate two distinct functional pools: the central pool of Lck would activate TCR-dependent signaling through its interaction with TCR-ζ, whereas the peripheral pool of Lck would down-regulate TCR-dependent signaling. Such a hypothesis is compatible with the dynamics of Lck translocation from the center of the synapse at very early time points to the periphery of mature synapses within 30 min of stimulation (50), a time when Lck-dependent negative feedback on T cell activation becomes operational. In this context, we propose a model in which Lck has a dual role: early in TCR engagement, Lck participates in the initiation and activation of downstream signaling pathways; this would be followed by inhibition of TCR-mediated signaling.

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References

NEGATIVE REGULATION OF T CELL ACTIVATION BY Lck


