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Bacterial Superantigens Bypass Lck-Dependent T Cell Receptor Signaling by Activating a Gα11-Dependent, PLC-β-Mediated Pathway

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Summary

The paradigm to explain antigen-dependent T cell receptor (TCR) signaling is based on the activation of the CD4 or CD8 coreceptor-associated kinase Lck. It is widely assumed that this paradigm is also applicable to signaling by bacterial superantigens. However, these bacterial toxins can activate human T cells lacking Lck, suggesting the existence of an additional pathway of TCR signaling. Here we showed that this alternative pathway operates in the absence of Lck-dependent tyrosine-phosphorylation events and was initiated by the TCR-dependent activation of raft-enriched heterotrimeric Gα11 proteins. This event, in turn, activated a phospholipase C-β and protein kinase C-mediated cascade that turned on the mitogen-activated protein kinases ERK-1 and ERK-2, triggered Ca^{2+} influx, and translocated the transcription factors NF-AT and NF-κB to the nucleus, ultimately inducing the production of interleukin-2 in Lck-deficient T cells. The triggering of this alternative pathway by superantigens suggests that these toxins use a G protein-coupled receptor as a coreceptor on T cells.

Introduction

Activation of the Src family tyrosine kinase Lck is one of the earliest events after engagement of the T cell receptor (TCR). Once activated, this kinase phosphorylates the immune receptor tyrosine-based activation motifs (ITAMs) on the ε, δ, γ, and ζ subunits of the TCR complex, providing the sites for recruitment and activation of the syk family kinase ZAP-70. In turn, ZAP-70 phosphorylates tyrosine residues in the cytoplasmic portion of the transmembrane adaptor LAT that act as docking sites for the assembly of a multimolecular signaling complex. These "signalosomes" trigger several downstream cascades that ultimately determine the translocation and activation of transcription factors leading to changes in gene expression that characterize activated T cells (Huang and Wange, 2004; Samelson, 2002). According to this paradigm, Lck is required to activate mature T cells with peptide-major histocompatibility complex (MHC) molecule complexes (Palacios and Weiss, 2004).

A substantial amount of the Lck expressed by mature T cells is associated with the CD4 or CD8 coreceptor (Weil and Veillette, 1996; Zamoyska et al., 2003). In this context, activation of Lck upon TCR binding to peptide-MHC complexes occurs after engagement of the CD4 or CD8 coreceptor molecules with MHC class II molecules or MHC class I molecules, respectively, and determines the pattern of early TCR signaling (Chau et al., 1998; Li et al., 2004; Madrenas et al., 1997). The molecular architecture that supports Lck-dependent T cell activation under these conditions involves the formation of dimers of TCR complexes, one engaged to an agonist peptide-MHC complex and the other to an endogenous peptide-MHC complex. The two engaged TCRs are bridged by a coreceptor molecule that facilitates Lck transactivation and the initiation of TCR signaling in response to conventional antigens (Krogsgaard et al., 2005).

There is experimental evidence suggesting that the T cell responses to bacterial superantigens (SAg) follow a different paradigm from that applicable to conventional antigens. For example, although most SAg are recognized in the context of MHC class II molecules, they are able to activate not only $CD4^+$ T cells but also $CD8^+$ T cells (Fuller and Braciale, 1998; Herrmann and MacDonald, 1993; Webb and Sprent, 1990), implying that the conventional CD4 and CD8 coreceptor assignment is not operational in these responses. Consistent with this observation, human T cell responses to some staphylococcal SAg such as enterotoxin B (SEB) or C-1 (SEC1) have been reported to be resistant to CD4 blockade and do not require expression of the CD4 coreceptor in the responding T cells (Bavari and Ulrich, 1995; Oyaizu et al., 1992). These observations suggest that human T cell responses to bacterial SAg do not require the CD4-associated Lck.

In addition to the cellular data, biochemical experiments indicate that bacterial SAg such as Staphylococcal enterotoxin E (SEE) can activate Lck-deficient, $CD4^{-\text{ or lo}}$ T cells (Criado and Madrenas, 2004; Rapecki and Allen, 2002; Yamasaki et al., 1997). In fact, Lck is not only dispensable but, as we and others have recently shown, it can also downregulate SAg-induced T cell activation by turning on a negative regulatory feedback on TCR signaling (Criado and Madrenas, 2004; Methi et al., 2005). Therefore, the ability of SAg to activate Lck-deficient, $CD4^-$ T cells suggests that the binding of these toxins to the TCR activates an additional pathway distinct from the canonical Lck-dependent cascade.

The nature of this alternative pathway is unknown. By using human primary T cells and T cell lines with well-characterized genetic deficiencies (Abraham and Weiss, 2004), we show that the SEE SAg bypasses the need for

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Lck and tyrosine phosphorylation of ZAP-70, LAT, and phospholipase C (PLC)-γ1 by activating the lipid raft-enriched, pertussis toxin-insensitive Gα11 proteins and its downstream target phospholipase C-β (PLC-β). This event triggers influx of Ca^{2+}, activation of protein kinase C (PKC) and of the mitogen-activated protein kinases (MAPK) ERK-1 and ERK-2, translocation of NF-AT and NF-κB transcription factors, and production of interleukin-2 (IL-2). This alternative signaling pathway explains why SEE and other bacterial SAg can overcome the lack of CD4 and Lck to activate human T cells and provides a clue to understand the biological responses to these bacterial toxins.

Results

The CD4 Coreceptor Is Dispensable for T Cell Responses to Bacterial SAgs

Most bacterial SAgs are recognized in the context of MHC class II molecules. However, they can activate both $CD4^+$ and $CD8^+$ T cells, suggesting that the CD4 coreceptor is not operational in these responses (Fuller and Braciale, 1998; Herrmann and MacDonald, 1993; Kappler et al., 1989; Webb and Sprent, 1990). To document the dispensability of CD4 in SAg-induced T cell responses, we determined the effect of CD4 blockade on the response of human peripheral blood T cells by using two different blocking antibodies against CD4 (OKT4 and RPA-T4). Human peripheral blood T cells responded to a set of staphylococcal and streptococcal SAgs (SEA, SEB, SEC-1, SEE, TSST-1, SME-Z, SpeI, SpeJ, SpeC) by producing substantial amounts of IL-2 (Figure 1A). More importantly, this response was not inhibited when blocking CD4 antibodies, either OKT4 or RPA-T4, were added to the culture. The blocking activity of these antibodies was confirmed in a mixed lymphocyte reaction ($p < 0.05$) (Figure 1B). Thus, bacterial SAgs do not require the CD4 coreceptor to activate T cells.

Lck Is Not Required for Activation of Peripheral Blood T Cells by SAg

The lack of requirement of CD4 function to respond to bacterial SAg suggests that Lck is not needed for this response. This conclusion was corroborated by several strategies. First, the response of primed human T cells to SEE and antigen-presenting cells (APCs) was preserved under conditions of Lck inhibition with PP2, a selective src kinase inhibitor (Figure 1C). In fact, as we reported (Criado and Madrenas, 2004) and recently confirmed (Methi et al., 2005), inhibition of Lck activity with PP2 enhanced such a response by blocking a still uncharacterized Lck-dependent negative feedback on TCR signaling. Similar results were obtained for another bacterial SAg from *Streptococcus pyogenes* (SME-Z) (data not shown). Although others have previously suggested that this response to SEE may be due to another src kinase expressed in T cells such as Fyn (Yamasaki et al., 1997), it is unlikely because PP2 inhibits Fyn and Lck with similar IC_{50} (Hanke et al., 1996) and because of the recent demonstration that Lck is needed for Fyn activation (Filipp and Julius, 2004). The possibility that the IL-2 response in the absence of Lck function was due to contaminating peptidoglycan (PGN), a TLR2 agonist present in gram-positive bacteria, was ruled out by the finding that PGN did not reverse the lack of IL-2 response to antibody-mediated TCR-CD3 ligation under conditions of Lck inhibition (Figure 1D). In fact, we observed that addition of PGN had an inhibitory effect on IL-2 response to OKT3, as previously reported (Nakagawa and Murai, 2003).

Next, we confirmed the Lck independence of T cell responses to SAg by using different SAg as well as human and mouse T cell lines and clones. The response to SEA by a mouse TCR Vβ3-expressing T cell clone and to SEE and SME-Z of a human Vβ8-expressing T cell line was not affected by the lack of functional Lck (Figure 2A). In contrast, Lck-deficient JCaM1.6 Jurkat T cells failed to produce IL-2 in response to plate bound anti-TCR and anti-CD28 (Figure 2B). We also tested the response of Lck-deficient T cells to anti-TCR Vβ (given the selective binding of SEE to TCR Vβ), but we found that none of the antibodies available were able to induce substantial T cell activation.

To further support the dispensability of Lck in the response to SEE-APCs stimulation, we performed Lck RNA interference. Transfection of Jurkat T cells with siRNA targeting Lck was able to knock down Lck protein amounts by 35% (the maximum percentage achieved in multiple attempts; $p < 0.05$). In these cells with decreased Lck expression, we did not observe any decrease in the amount of IL-2 produced upon stimulation with SEE-APCs (Figure 2C).

Activation of Lck-deficient T cells by SEE was further documented by upregulation of CD69 on the surface of T cells (Figure 2D) and by the formation of immunological synapses (Figure 2E). It is important to note that the response to SEE by Lck-deficient T cells required TCR-mediated recognition of the bacterial SAg because the Jurkat JRT3 subline, which lacks TCR β expression (Weiss and Stobo, 1984), did not respond to SEE (Figure 2F), although it responded to PMA and ionomycin (data not shown). To further demonstrate the TCR dependence of the response to SEE of Lck-deficient T cells, we generated ultrapure wild-type SEE as well as a mutant form of this SAg in which the TCR-interacting tyrosine residue at position 205 was mutated to an alanine (Y205A). As control, we generated a SEE mutant at a site distal to the TCR contact interface (K148A). When the responses to these SAgs were tested, we observed that the Y205A SEE failed to induce IL-2 production by Lck-deficient T cells, whereas both the wild-type SEE and the K148A SEE induced substantial IL-2 production by these cells (Figure 2G). Together, these results confirm with multiple approaches that the response of human primary T cells and T cell lines to SEE and other SAgs does not require the canonical Lck-dependent signaling pathway (Criado and Madrenas, 2004; Rapecki and Allen, 2002; Yamasaki et al., 1997).

Bacterial SAg Bypass the Lck Requirement by Triggering an Alternative Signaling Pathway

The finding of T cell activation through the TCR in response to a biologically relevant stimulus in the absence of Lck provided an excellent opportunity to characterize alternative TCR signaling pathways. Because one of the earliest events upon TCR-induced Lck activation is the recruitment and phosphorylation of ZAP-70 (Iwashima

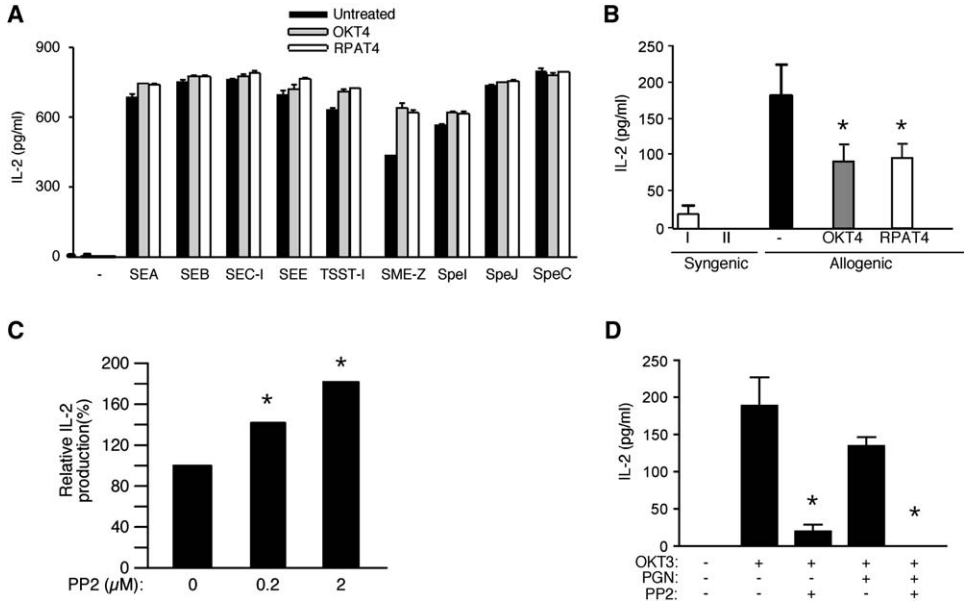

Figure 1. CD4-Independent and Lck-Independent T Cell Activation by Bacterial SAgs

(A) PBMC (10^5 cells) were stimulated with 0.01 ng/ml of SEA, SEB, SEC-1, SEE, or TSST or 0.1 ng/ml of SEMZ, SpeI, SpeJ, or SpeC in the presence or absence of 10 μg/ml of either OKT4 or RPA-T4 antibody. 48 hr later, supernatants were collected and IL-2 was measured by ELISA. Results (mean ± standard deviation of triplicates) are representative of three independent experiments.

(B) MLR of PBMC (10^5 cells) stimulated with the same number of syngeneic or allogeneic irradiated PBMC (5000 Rads) in the presence or absence of 10 μg/ml of either OKT4 or RPA-T4 antibody. 4 day culture supernatants were collected and assessed for IL-2 by ELISA. The effect of CD4 blockade on the allogeneic MLR was statistically significant (*$p < 0.05$). Results (mean ± standard deviation of triplicates) are representative of two independent experiments.

(C) Human T cell blasts were prepared from peripheral blood lymphocytes as indicated in the Experimental Procedures and stimulated with monocyte-derived dendritic cells and SEE in the presence or absence of increasing concentrations of the selective src kinase inhibitor PP2. IL-2 production was measured by ELISA of overnight culture supernatants. Relative IL-2 production was calculated in relation to that of SEE-stimulated T cells in the absence of PP2 (considered as 100%). *$p < 0.05$.

(D) Staphylococcal peptidoglycan (PGN) does not restore IL-2 production by human peripheral blood T cells lacking Lck activity. Human peripheral blood mononuclear cells (10^5 cells/well in triplicate for each group) were isolated from a normal volunteer and stimulated with OKT3 in the absence or presence of the Lck-inhibitor PP2 (10 μM) and PGN (10 μg/ml). IL-2 production in 24 hr cultures was measured by ELISA. Results (mean ± standard deviation of triplicates) are representative of three independent experiments. *$p < 0.05$.

et al., 1994), we examined the phosphorylation of ZAP-70 upon SEE-APCs stimulation in the Jurkat E6.1 T cell line and its Lck-deficient derivative JCaM1.6. We found that stimulation with SEE-APCs caused an increase in phosphorylation of ZAP-70 in E6.1 T cells but not in JCaM1.6 T cells (Figure 3A). This result is in line with our previous finding that ZAP-70-deficient T cells respond to SEE (Shan et al., 2001). The absence of Lck activity in JCaM1.6 T cells was confirmed by measuring Lck kinase activity on the exogenous substrate enolase (Figure 3B).

The paradox raised by the IL-2 response to SEE in Lck-deficient T cells in the absence of ZAP-70 phosphorylation (a reflection of its activation) could have been due to low amounts of phosphorylation below detection rather than true lack of phosphorylation of this kinase. To rule out this possibility, we examined several other steps downstream of ZAP-70 activation that would have been amplified by ZAP-70 activation. We looked at phosphorylation of one of the ZAP-70 substrates, the linker for activated T cells (LAT), a key event for the assembly of signalosomes connecting the TCR-proximal events to downstream signaling pathways (Chau and Madrenas, 1999; Madrenas, 2003). Consistent with the lack of ZAP-70 activation, we found that SEE-APCs did not induce phosphorylation of LAT (Figure 3C). We also examined tyrosine phosphorylation (activation) of PLC-γ1, a critical enzyme that directly binds phosphorylated LAT and mediates hydrolysis of inositol phospholipids into diacylglycerol and inositol-(1, 4, 5)-trisphosphate (IP3) (Yablonski et al., 1998; Yablonski and Weiss, 2001). Again, we failed to detect tyrosine phosphorylation of PLC-γ1 in response to SEE by Lck-deficient T cells (Figure 3D). Together, these findings established that TCR signaling upon SEE recognition can take place through a pathway other than the canonical Lck-dependent pathway.

The Alternative Signaling Pathway Triggered by SAg Leads to Activation of ERK and PKC and to Ca^{2+} Influx

To characterize this alternative pathway, we first examined the effect of different kinase inhibitors on the response of Lck-deficient T cells to SEE. We found that the MEK inhibitor PD98059 inhibited the IL-2 response to SEE by Lck-deficient T cells (Figure 4). In addition, we observed that the PKC inhibitor G06850 markedly inhibited the response of Lck-deficient T cells to SEE (Figure 4). Similar results were obtained with other PKC inhibitors (Rö-31-8220 and Rottlerin, data not shown). In contrast, inhibition of PI3K by LY294002 did not have any effect on the SEE-induced IL-2 production by Lck-deficient T cells (Figure 4).

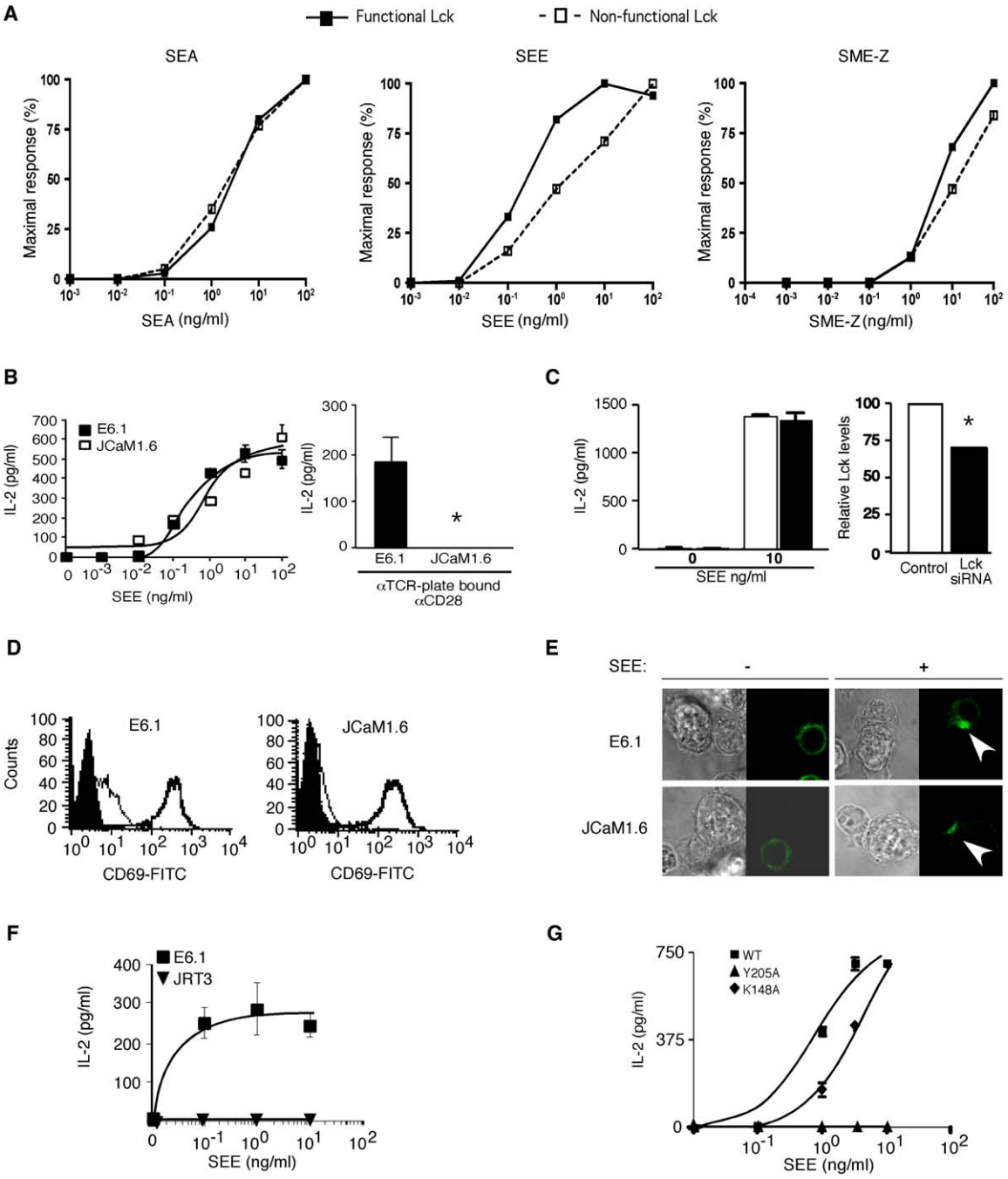

Figure 2. Lck Is Not Required for IL-2 Production by T Cells in Response to SEE

(A) Lck activity is not required for T cell responses to different superantigens. Untreated or PP2-treated (10 μM) mouse 3C6 T cells (expressing TCR Vβ3) were stimulated with the indicated concentrations of Staphylococcal Enterotoxin A (SEA), and APC and IL-2 production was measured by ELISA after 24 hr of culture. Similar experiment was performed with Jurkat E6.1 T cells and Lck-deficient JCaM1.6 Jurkat T cells (both expressing TCR Vβ8) with SEE or with the Streptococcal superantigen SME-Z and LG-2 APC. Results (mean ± standard deviation of triplicates) are representative of at least two independent experiments.

(B) Parental Jurkat T cells (E6.1) and their Lck-deficient counterparts (JCaM1.6) were stimulated with increasing concentrations of SEE and APC (left) or plate bound anti-TCR plus anti-CD28 (5 μg/ml) (right). After 24 hr, culture supernatants were collected and assayed for IL-2 production by ELISA. Results (mean ± standard deviation of triplicates) are representative of four independent experiments. *$p < 0.05$.

(C) E6.1 Jurkat T cells were transfected either with Lck siRNA or with nucleotide control. 48 hr after transfection, T cells were collected and activated with APCs and SEE (10 ng/ml). 24 hr supernatants were assessed for IL-2 production by ELISA. Intracellular Lck was determined by flow cytometry with the DAKO IntraStaining kit (DAKO A/S, Glostrup, Denmark). Results (mean ± standard deviation of triplicates) are representative of three independent experiments. *$p < 0.05$.

(D) Jurkat E6.1 T cells and JCaM1.6 were stimulated with APC in the presence (thick line) or absence (thin line) of SEE (10 ng/ml) for 16 hr. Cells were harvested, stained with FITC-labeled CD69 mAb, and analyzed by flow cytometer. Basal CD69 expression on Jurkat cells is shown by filled profile.

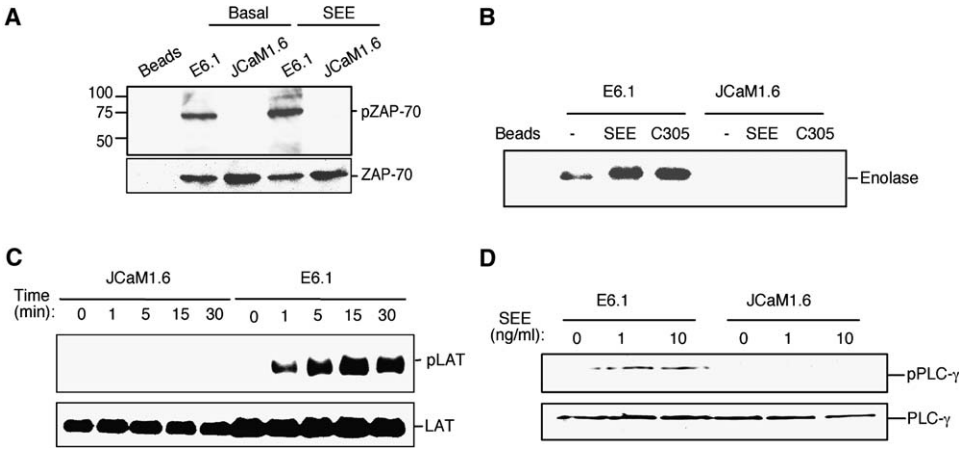

Figure 3. Lack of Early Tyrosine Phosphorylation Signaling Events upon SEE Recognition by Lck-Deficient T Cells

(A) Tyrosine phosphorylation of ZAP-70 as shown by a phosphotyrosine immunoblot of ZAP-70 immunoprecipitates from E6.1 and JCaM1.6 Jurkat T cells (10^7/lane) activated by APC in the presence or absence of SEE (10 ng/ml) for 10 min; ZAP-70 immunoblotting confirmed equal loading (bottom).

(B) Lck kinase assay of Lck immunoprecipitates from E6.1 and JCaM1.6 Jurkat T cells (10^7/sample) activated for 10 min with APC in the presence or absence of SEE (10 ng/ml) or anti-TCR C305. Samples were incubated with enolase (160 μg/ml) in the presence of [γ^{32}P]ATP (10 μCi/sample), resolved on 8% SDS PAGE, and analyzed with a PhosphorImager.

(C) Parental Jurkat T cells (E6.1) and their Lck-deficient counterparts (JCaM1.6) were stimulated with APC and SEE (10 ng/ml) for the indicated times. Lysates were immunoprecipitated with LAT antisera and sequentially blotted for phosphotyrosine and for LAT (bottom).

(D) Lysates from E6.1 and JCaM1.6 Jurkat T cells stimulated for 10 min with APC and SEE at the indicated concentrations were used for immunoprecipitation of PLC-γ1 and sequentially blotted for phosphotyrosine and for PLC-γ1. Results in this figure are representative of at least three different experiments.

Consistent with the above functional results, we found that Lck-deficient T cells responded to SEE with slightly less but still substantial ERK-1 and ERK-2 activation (Figure 5A), a remarkable finding when considering that the current paradigm of TCR signaling makes MAPK activation contingent on tyrosine phosphorylation of ZAP-70, of LAT, and of PLC-γ1 (Finco et al., 1998; Martelli et al., 2000; Williams et al., 1999). We also looked at events that link ERK-1 and ERK-2 activation with IL-2 production in Lck-deficient T cells. Because IL-2 production requires Ca^{2+} influx, we examined whether Lck-deficient T cells underwent Ca^{2+} fluxing in response to SEE. We found that stimulation with SEE-APCs induced marked intracellular Ca^{+2} mobilization in Lck-deficient T cells. Such response was similar in magnitude to that displayed by Lck-repleted T cells even though JCaM1.6 T cells showed an 85% reduction in Ca^{+2} mobilization when stimulated with ionomycin (Figure 5B and inserts).

Next, we determined the activation state and translocation of NF-AT and NF-κB, two transcription factors involved in the upregulation of IL-2 gene transcription. Nuclear and cytoplasmic fractions of resting and SEE-stimulated E6.1 and JCaM1.6 T cells were prepared and blotted for NF-AT. We found that stimulation with SEE-APCs induced an increase of dephosphorylated (activated) NF-AT in the nucleus in Lck-repleted T cells as well as in Lck-deficient T cells (Figure 5C). To assess NF-κB activation, we determined the phosphorylation of the cytosolic inhibitor of this transcription factor (IκB), an event linked to activation of this transcription factor. We found that activation of this transcription factor occurred to a similar magnitude in Lck-deficient T cells in response to SEE stimulation (Figure 5D). Together, these data demonstrate that T cell activation by SEE involves an additional pathway from the TCR that is distinct from the canonical Lck-dependent pathway and that can trigger by itself, in the absence of such canonical pathway, the downstream responses required for T cell activation, i.e., it can activate and translocate transcription factors required for IL-2 gene expression.

SEE Triggers a Gα11 and PLC-β-Dependent Pathway

Because SEE triggered Ca^{2+} influx and PKC activation, we hypothesized that a phospholipase C (PLC) other than PLC-γ1 had to be involved in such an alternative pathway of TCR signaling because no PLC-γ1 activation was detected under these conditions. A candidate is PLC-β, a PLC expressed by T lymphocytes (Miscia et al., 1999). To assess the role of PLC-β, we utilized the selective inhibitor of this enzyme U73122 and its control compound U73343 and analyzed their effects

(E) Jurkat E6.1 and JCaM1.6 cells were stimulated for 30 min with APC in the presence or absence of SEE (100 ng/ml), stained with anti-CD3-FITC (green signal), and analyzed by confocal microscopy. White arrowheads point to putative immunological synapses at T cell-APC interface; confocal figures are representative of at least 40–50 putative synapses for each group, as previously reported.

(F) Jurkat T cells E6.1 and their TCR β-deficient counterpart cells (JRT3) were stimulated with increasing concentrations of SEE and APC. After 24 hr, culture supernatants were collected and assayed for IL-2 production by ELISA. Results (mean ± standard deviation of triplicates) are representative of three independent experiments.

(G) Lck-deficient JCaM1.6 Jurkat T cells were stimulated with APC and wild-type or mutant SEE molecules at the indicated concentrations as above, and IL-2 production was measured by ELISA. Results (mean ± standard deviation of triplicates) are representative of three independent experiments.

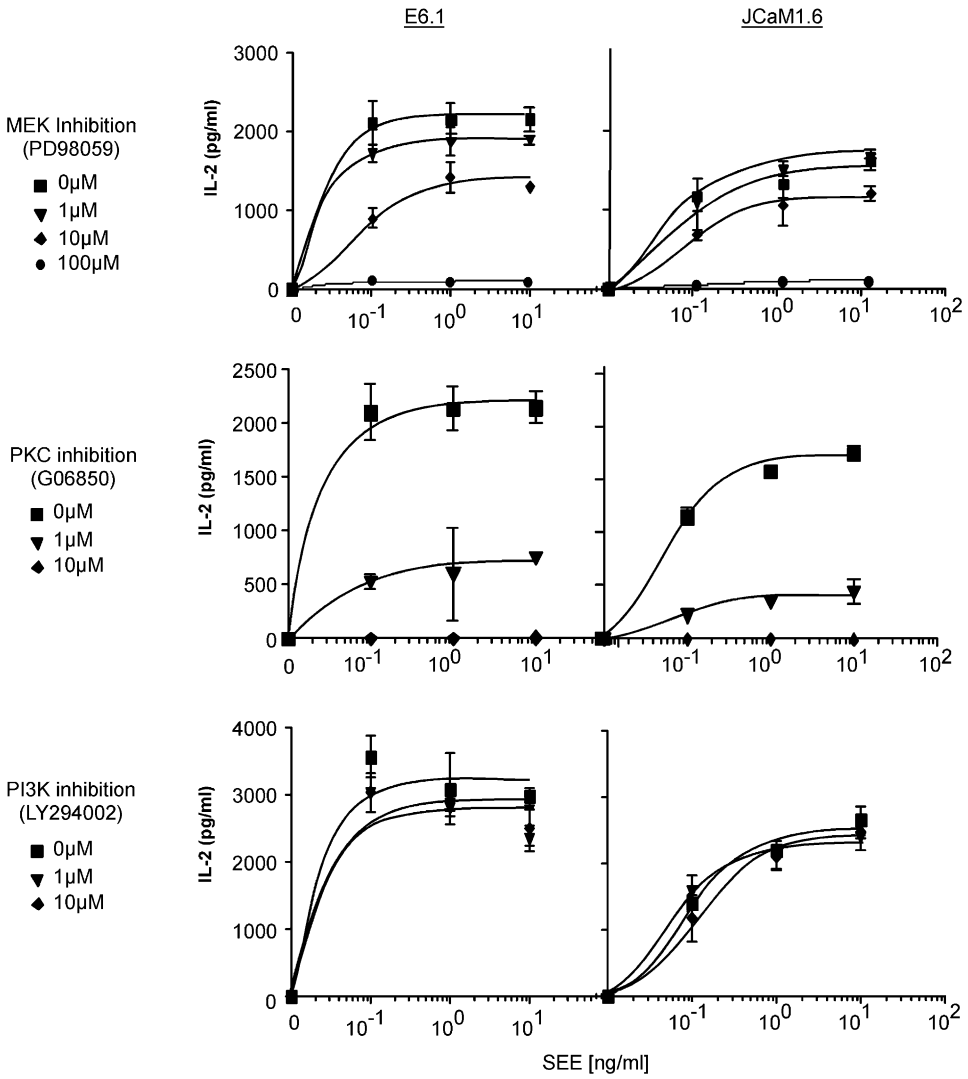

Figure 4. Activation of Lck-Deficient T Cells by SEE Utilizes a PKC- and MEK-Dependent Signaling Pathway

Parental Jurkat T cells (E6.1) and their Lck-deficient counterparts (JCaM1.6) were stimulated with APC and increasing concentrations of SEE in the presence of the indicated concentrations of the MEK inhibitor PD98059 (top), of the PKC inhibitor GO6850 (middle), and of the PI3K inhibitor LY294002 (bottom) for 24 hr. Supernatants were then collected and assessed for IL-2 production by ELISA. Results for each point represent mean ± SEM. The decrease in IL-2 production observed for PD98059 and for LY294002 treatments were statistically significant ($p < 0.05$). Results are representative of at least three different experiments.

on ERK-1 and ERK-2 activation induced by SEE in Lck-deficient T cells. We found that pharmacological inhibition of PLC-β resulted in an almost complete blockade of ERK-1 and ERK-2 activation in Lck-deficient T cells in response to SEE (Figure 6A). Such an effect was also observed in Lck-repleted T cells although the magnitude of this effect was not as intense (data not shown).

Next, we investigated the effect of PLC-β inhibition in primary human T cells and found that SEE-induced T cell responses were inhibited when the cells were treated with the PLC-β inhibitor U73122. We observed that the responses to high-order CD3 crosslinking with OKT3 were also inhibited by U73122, a finding that could be explained by residual activity of this compound on PLC-γ1. To segregate the effect of inhibition of PLC-β from that of PLC-γ1 inhibition, we used the Lck inhibitor PP2. Lck is responsible for phosphorylation and activation of PLC-γ1. We observed that OKT3-induced ERK-1 and ERK-2 activation was blocked, whereas SEE-induced activation of ERK-1 and ERK-2 was still detectable (Figure 6B), thus corroborating that phosphorylation of PLC-γ1 is not required in SEE-induced T cell response. To further confirm the involvement of PLC-β in this response, we knocked down PLC-β_1 by RNA interference in the Lck-deficient JCaM1.6 T cells. Under conditions of 60% to 75% down-regulation of PLC-β, there was a significant decrease in the amount of IL-2 produced in response to SEE-APCs compared with the control ($p < 0.05$) (Figure 6C). Western blots documented the selective decrease in PLC-β by RNA interference whereas total ERK-1 and ERK-2 levels were not affected (Figure 6C, right).

PLC-β is regulated by the pertussis toxin-insensitive Gαq11 family of G proteins (Philip et al., 2002). These G proteins have been previously linked to TCR signaling after high degree of crosslinking of CD3 (Stanners et al., 1995). Therefore, we hypothesized that these G proteins

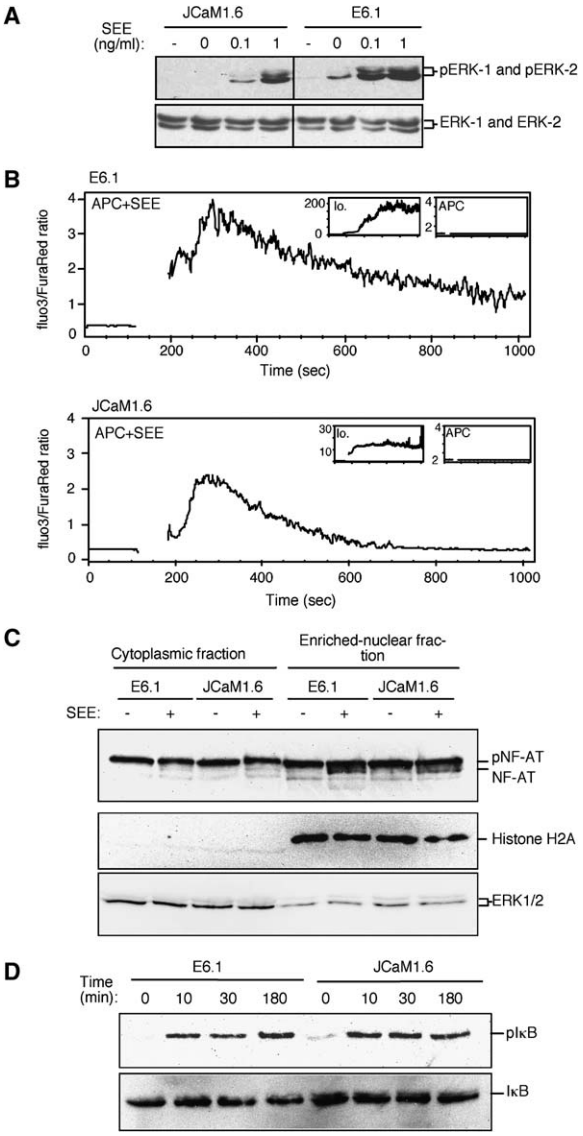

Figure 5. SEE-Induced Activation of Lck-Deficient T Cells Triggers ERK-1 and ERK-2 Activation, Ca^{2+} Influx, and Activation and Translocation of NF-AT and NF-κB

(A) Parental Jurkat T cells (E6.1) and their Lck-deficient counterparts (JCaM1.6) were stimulated with APC and increasing concentrations of SEE for 10 min. Whole-cell lysates were prepared and sequentially immunoblotted for active ERK-1 and ERK-2 and total ERK-1 and ERK-2 content.

(B) Jurkat E6.1 and JCaM1.6 T cells were loaded with fluo-3AM and FuraRedAM. Baseline fluorescence ratio (fluo3/FuraRed) was measured for 2 min prior to the administration of SEE-loaded APC, and reading of the fluorescence ratio was acquired for an additional 15 min. APC without SEE culture was used as a negative control (right panel of inlets), and ionomycin (2 μg/ml) was used as a positive control (left panel of inlets).

(C) Jurkat E6.1 and JCaM1.6 T cells were stimulated with SEE (10 ng/ml) and APC for 10 min. Cytoplasmic fractions and enriched-nuclear fractions were prepared for these samples as described in the Experimental Procedures. NF-AT activation in these fractions was evaluated by blotting for NF-AT and assessing dephosphorylated NF-AT in the nucleus as indicated by appearance of lower NF-AT band. Purity of the cytoplasmic and enriched nuclear fractions was confirmed by immunoblotting for total ERK-1 and ERK-2 and histone H2A, respectively.

(D) Jurkat E6.1 and JCaM1.6 T cells were stimulated with SEE (10 ng/ml) and APC for the indicated times. Whole-cell lysates were prepared and immunoblotted for phospho-IκB, which reflects NF-κB activation. Total IκB immunoblotting confirmed equal loading for each lane. Results in this figure are representative of at least three independent experiments.

are activated by SEE, and this would explain the activation of downstream events in Lck-deficient T cells. First, we confirmed that Gα11 is expressed by Lck-deficient Jurkat T cells. We fractionated JCaM1.6 T cells and prepared soluble and lipid rafts fractions of these cells because G proteins are enriched in these microdomains, and we immunoblotted them for Gα11. JCaM1.6 T cells expressed Gα11, and as reported for other G proteins (Chini and Parenti, 2004), it localized within lipid rafts (Figure 7A). The quality of the raft fractionation procedure was confirmed by blotting for ERK-1 and ERK-2, which partition in soluble fractions, and GM1, which partitions in lipid rafts.

Next, we introduced a dominant-negative form for Gα11 in Jurkat T cells with the idea that interference with the function of this protein would inhibit the response of T cells to SEE. We used the G208A Gα11 cDNA construct that has been extensively characterized as a selective blocker of endogenous Gα11 proteins (Stanners et al., 1995). We found that T cells stably transfected with the Gα11 dominant-negative construct failed to respond to SEE with activation of ERK-1 and ERK-2 (Figure 7B). In contrast, the same cells transfected with a neomycin control gene responded normally. Similar results were obtained upon transient cotransfection of JCaM1.6 T cells (data not shown).

The involvement of Gα11 proteins in the T cell response to bacterial SAg was further corroborated by the observation that the early response of Lck-deficient T cells to SEE was not inhibited by pertussis toxin (Figure 7C), consistent with the fact that Gαq11 proteins are insensitive to this toxin. Of interest, late responses such as IL-2 production did show slight sensitivity to pertussis toxin (Figure 7C), consistent with the multiple coupling of G protein-coupled receptors with time (Hermans, 2003). We also knocked down Gα11 protein by RNA interference in Lck-deficient T cells. Despite the fact that the drop in Gα11 protein by this technique was consistently small, we observed that T cells with decreased Gα11 expression had significantly lower IL-2 production in response to SEE than the control T cells ($p < 0.05$) (Figure 7D). Western blots documented the selective decrease in Gα11 by RNA interference, whereas total ERK-1 and ERK-2 levels were not affected (Figure 7D, right). Together, our results demonstrate that SEE can bypass the requirement for Lck in TCR-dependent activation via a Gα11-dependent pathway that leads to PLC-β activation, influx of Ca^{2+}, activation and translocation of NF-AT and NF-κB, and IL-2 production.

Discussion

It is widely assumed that TCR signaling by bacterial SAg follows the canonical Lck-dependent pathway used by conventional peptide-MHC complexes and by antibody-mediated crosslinking of the TCR complex and is characterized by the formation of signalosomes on phosphorylated LAT. However, emerging evidence

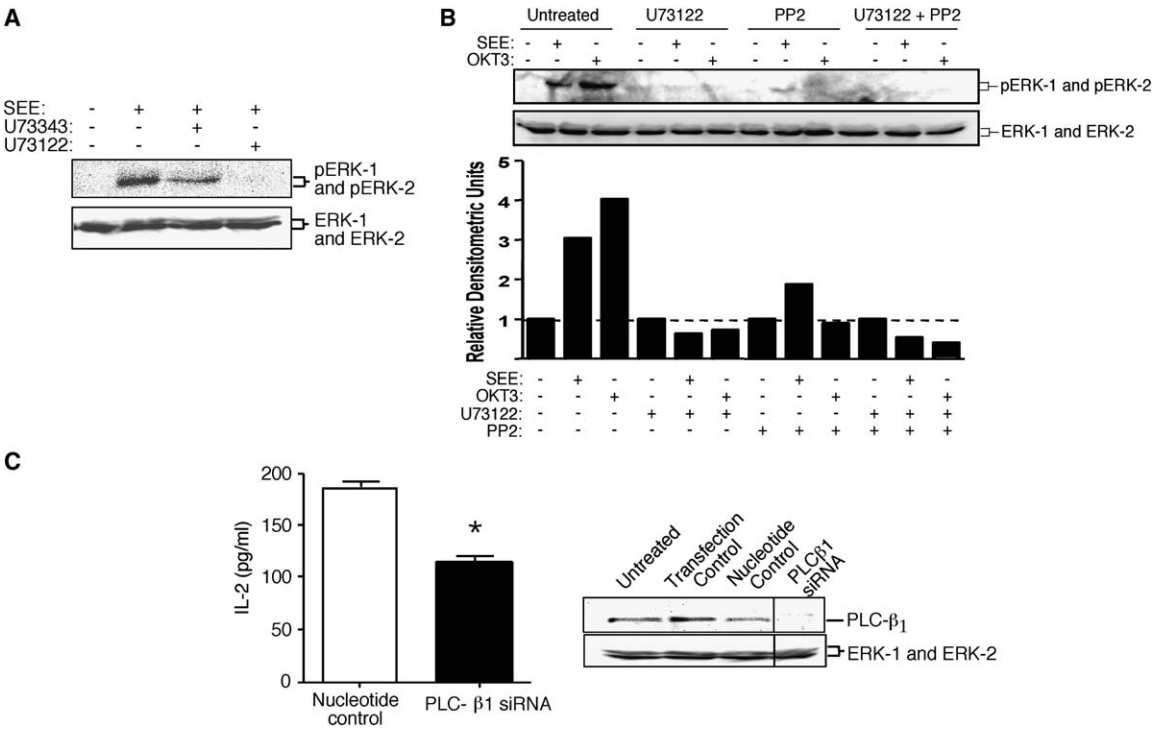

Figure 6. SEE Activates a PLC-β-Signaling Pathway

(A) JCaM1.6 Jurkat T cells were incubated for 1 hr in the presence of the PLC-β inhibitor (U73122) (10 μM) or its inactive analog (U73343) prior to stimulation with APC and SEE (10 ng/ml) for 10 min. Whole-cell lysates were prepared and sequentially immunoblotted for active ERK-1 and ERK-2 and total ERK-1 and ERK-2 (to confirm equal loading).

(B) PBMC were incubated for 1 hr in the presence of the PLC-β inhibitor U73122 (10 μM) or the src kinase PP2 (20 μM) or the combination of both prior to stimulation with SEE (10 ng/ml) for 10 min. Whole-cell lysates were prepared and sequentially immunoblotted for active ERK-1 and ERK-2 and total ERK-1 and ERK-2 (to confirm equal loading). Densitometric analysis of dually phosphorylated ERK-1 and ERK-2 is shown in the bottom panel. Background signal level is shown by the dashed line.

(C) JCaM1.6 Jurkat T cells were transfected either with PLC-β_1 siRNA or nucleotide control. 24 hr after transfection, cells were collected and activated with SEE (1 ng/ml). 24 hr supernatants were assessed for IL-2 production by ELISA. PLC-β_1 expression was determined in whole-cell lysates by immunoblot, and equal loading was confirmed by ERK-1 and ERK-2 blotting. Results (mean ± standard deviation of triplicates) are representative of five independent experiments. *$p < 0.05$.

indicates that bacterial SAg can also use an alternative pathway. Such evidence includes the ability of these bacterial toxins (but not of peptide-MHC ligands or anti-CD3) to fully activate T cells lacking Lck, and also T cells lacking ZAP-70 (Shan et al., 2001), and to do so without any tyrosine phosphorylation events triggered by a src kinase, ruling out a contribution of Fyn, the other src kinase expressed in some mature T cells. The results presented here demonstrate that the alternative TCR signaling pathway triggered by SEE in the absence of Lck involves the activation of Gα11 and its downstream target PLC-β. The activity of PLC-β leads to Ca^{2+} influx and to activation of PKC and ERK-1 and ERK-2. These events then converge in the activation and translocation of transcription factors required for de novo IL-2 gene transcription and IL-2 production that defines full T cell activation. Importantly, both the canonical and the alternative pathways crosstalk and can work in concert in mature T cells, but activation of the Gα11-PLC-β pathway is necessary and sufficient to activate T cells.

A role for G proteins, and in particular for those containing the Gα11 subunit, has been previously suggested for TCR signaling and T cell activation (Coffield et al., 2004; Ohmura et al., 1992; Stanners et al., 1995), and mice deficient for the Gα11 regulator RGS2 have impaired T cell proliferation and IL-2 production (Oliveira-Dos-Santos et al., 2000). In addition, Gαq-coupled receptors can trigger NF-AT translocation (Boss et al., 1996) and NF-κB activation (Liu and Wong, 2004), both key events for the initiation of IL-2 gene transcription. However, the biological implications of these observations have remained uncertain because they were generated in artificial systems (antibody-mediated crosslinking of the TCR-CD3 complex) or were masked by the presence of a fully operational Lck-dependent pathway. The evidence presented here provides a biologically relevant condition in which a G protein-dependent pathway emanating from the TCR leads to full activation of mature T cells in the absence of confounding Lck-dependent events.

How SEE activates Gα11 remains to be determined. One possibility is that TCR engagement by SEE induces a high degree of TCR crosslinking as anti-CD3 do, and this leads to G protein activation by aggregation of lipid rafts where these proteins are enriched (Chini and Parenti, 2004; Ohmura et al., 1992; Stanners et al., 1995). Alternatively, bacterial SAg may concomitantly bind to the TCR and to a Gα11 protein-coupled surface molecule that then acts as a coreceptor and, through its aggregation, facilitates the activation of Gα11. This possibility is consistent with the observation that binding of

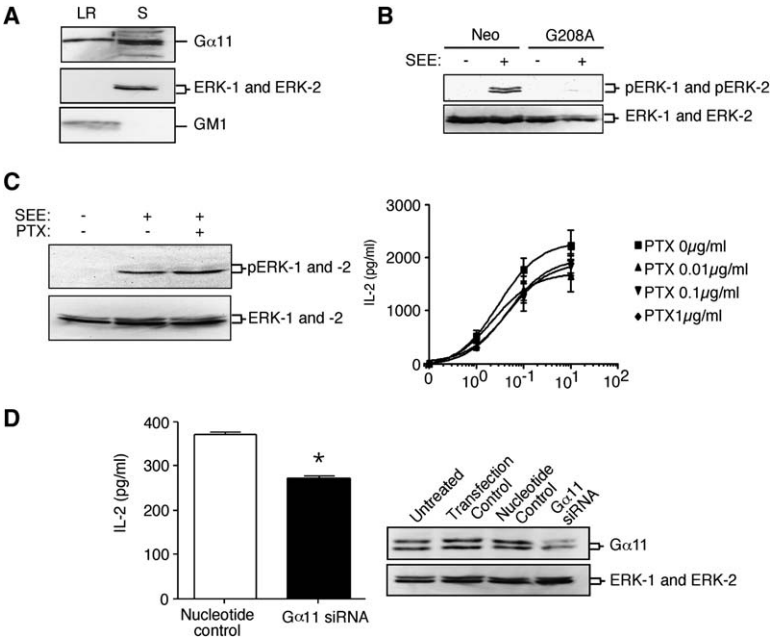

Figure 7. SEE Activates Pertussis Toxin-Insensitive Gα11 Proteins

(A) Gα11 is expressed by Lck-deficient JCaM1.6 T cells. Lipid rafts (LR) and detergent-soluble (S) fractions were isolated from resting JCaM1.6 Jurkat T cells and immunoblotted for Gα11. Quality of the LR and S fractions was assessed by immunoblotting for GM1 and ERK-1 and ERK-2 as raft and cytosolic representative molecules, respectively.

(B) A Gα11 dominant-negative blocks the T cell response to SEE. Whole-cell lysates from stable E6.1 transfectants for Neomycin control vector (Neo) or Gα11 dominant-negative mutant (G208A) were prepared and sequentially immunoblotted for active ERK-1 and ERK-2 and total ERK-1 and ERK-2.

(C) The G protein activated by SEE is pertussis toxin insensitive. Lck-deficient JCaM1.6 Jurkat cells were incubated for 1 hr in the presence of PTX (1 μg/ml) prior to stimulation with APC and SEE (10 ng/ml). Whole-cell lysates were prepared and sequentially immunoblotted for active ERK-1 and ERK-2 and for total ERK-1 and ERK-2. Lck-deficient Jurkat cells (JCaM1.6) were incubated for 1 hr in the presence of PTX at the indicated concentrations prior to stimulation with APC and increasing concentrations of SEE for 24 hr. Supernatants were collected and assessed for IL-2 production by ELISA. Results represent mean ± SEM of three experiments.

(D) Effect of Gα11 RNA interference on SEE-induced T cell activation. JCaM1.6 Jurkat T cells were transfected either with Gα11 siRNA or nucleotide control. 24 hr after transfection, cells were collected and activated with APCs and SEE (0.01 ng/ml). 24 hr supernatants were assessed for IL-2 production by ELISA. Gα11 expression was determined in whole-cell lysates by immunoblot and equal loading confirmed by ERK-1 and ERK-2 blotting. Results for each point represent mean ± SEM and are representative for three different experiments. *p < 0.05.

the bacterial toxin to the TCR is still required for activation of this alternative pathway. As potential coreceptors, we thought of the chemokine receptors CXCR4 and CCR5, for which a costimulatory function during T cell activation, through Gα11, has been recently claimed (Molon et al., 2005). Of interest, these authors used an experimental model based on SEE-induced activation of Lck-repleted Jurkat T cells. However, chemokine receptors are not the putative coreceptors because signaling through CXCR4 requires Lck and Lck-dependent ZAP-70 phosphorylation (Inngjerdingen et al., 2002; Kremer et al., 2003), because JCaM1.6 T cells do not express CCR5, and because antibody blockade of their function did not prevent the response of Lck-deficient T cells to SEE (C.B. and J.M., unpublished observations). We also ruled out the involvement of other conventional G protein-coupled receptors for which primary association with Gαq/11 and subsequent multiple G protein coupling have been reported (e.g., adrenergic receptors, muscarinic receptors), via pharmacological antagonists (C.B. and J.M., unpublished observations). A third type of SAg coreceptor candidate is lipid raft-enriched gangliosides because it has been previously reported that SEB binds membrane glycosphingolipids (Chatterjee and Jett, 1992) and because ligation of GM1 can trigger some signaling events including ERK-1 and ERK-2 activation (Duchemin et al., 2002; Janes et al., 1999; Kiyokawa et al., 2005; Singleton et al., 2000). Experiments are underway to explore the role of gangliosides in the response to bacterial SAg.

Finally, our data help to explain the paradoxes regarding human T cell responses to some SAg. The differentiation of T cells along Th1 and Th2 pathways is, in part, determined by the pattern of TCR signaling (Madrenas, 2003). It has been proposed that Th1 differentiation is relatively Lck independent while Th2 differentiation requires Lck activity (al-Ramadi et al., 1996; Yamashita et al., 1998). SEE-induced immune responses in vivo have been mostly associated with a Th1 profile (Gehring et al., 1998; Hamel et al., 1995), consistent with the dispensability of Lck for immune responses to bacterial SAg. Also, the CD4 independence of T cell responses to some bacterial SAg (Herrmann and MacDonald, 1993; Webb and Sprent, 1990) and the ability to activate $CD8^+$ T cells despite these toxins being recognized in the context of MHC class II binding (Bavari and Ulrich, 1995; Oyaizu et al., 1992) would be a reflection of the ability of these toxins to bypass the requirement for the conventional CD4 or CD8 coreceptor and its associated Lck kinase to initiate TCR signaling.

Experimental Procedures

Cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers by Ficoll gradient centrifugation (Ficoll-Paque Plus, Amersham Biosciences, Piscataway, NJ). T cell blasts were generated from PBMCs by treatment with PMA (1 ng/ml) and ionomycin (100 ng/ml) as previously described (Criado and Madrenas, 2004). Monocyte-derived dendritic cells (MD-DC) were generated from PBMCs by treatment with GM-CSF (1000 U/ml) and IL-4 (1000 U/ml) as previously described (Criado and Madrenas, 2004). Human cells were obtained and processed in compliance with research protocols approved by the Office of Research Ethics at the University of Western Ontario. The Jurkat E6.1 T cell line and the Lck-deficient Jurkat subline JCaM1.6 were purchased from the American Tissue Culture Collection (Manassas, VA) and maintained in RPMI + 10% FCS. The lymphoblastoid B cell line LG2 was provided by Dr. Eric Long (National Institutes of Health, Rockville, MD) and maintained in RPMI + 10% FCS. Jurkat T cells were transfected

with a Gα11 wild-type cDNA (wt) or with a G208A Gα11 dominant-negative cDNA (Jurkat Gα11 G208A) or with a control plasmid (Neo Jurkat) as described (Stanners et al., 1995) and culture in RPMI + 10% FCS as above except for the inclusion of 500 μg/ml geneticin for selection. The mouse $CD4^+$ Th1 clone 3C6 (which expresses TCR Vβ3) has been previously described (Madrenas et al., 1995, 1997).

Antibodies and Reagents

The following monoclonal antibodies (mAb) were used in these experiments: anti-CD3 UCHT-1, anti-CD4 RPA-T4, and anti-CD28 CD28.2 from eBioscience (San Diego, CA); anti-CD4 OKT4 from Ortho Biotech Inc; anti-active ERK-1 and ERK-2 E10, anti-PLCγ B-6-4, IκB-ε polyclonal antibody, phospho-IκB-α (Ser32) polyclonal antibody, and Histone H2A polyclonal antibody from Cell Signaling Technology (Beverly, MA); anti-phosphotyrosine 4G10 was kindly provided by Dr. B. Drukker (Oregon Health Sciences University, Portland, OR). An ERK-1 and ERK-2 rabbit polyclonal antiserum was purchased from StressGen (Victoria, BC, Canada). LAT rabbit polyclonal antibody and ZAP70 rabbit polyclonal antiserum were purchased from Upstate Biotechnology (Lake Placid, NY). FITC-labeled anti-CD69, anti-NFAT-1 clone 1, anti-CXCR4 clone 12G5, and anti-CCR5 clone 2D7 were obtained from BD Biosciences (San Diego, CA). Goat anti-rabbit Ig HRPO conjugated was purchased from BioRad (Hercules, CA). Sheep anti-mouse Ig HRPO conjugated was obtained from Amersham Pharmacia Biotech Inc. The anti-Gα11 mAb clone D-17 and the anti-PLC-β1 mAb clone D-8 were purchased from Santa Cruz Biotechnology, Inc. The anti-TCR IgM antibody C305 was kindly provided by Dr. A. Weiss (University of California at San Francisco) (Weiss and Stobo, 1984). The selective Src-tyrosine kinase inhibitor PP2, the PKC inhibitor Bisindolylmaleimide I (Gö 6850), the MAP kinase kinase (MEK) inhibitor PD98059, and the phosphatidylinositol 3-kinase (PI3-kinase) inhibitor LY294004 were all purchased from Calbiochem. The PLC-β inhibitor U-73122 and its inactive analog U-73343 were purchased from Sigma-Aldrich (Oakville, ON). Pertussis toxin (PTX) was purchased from List Laboratories (Campbell, CA). Staphylococcal enterotoxin E was purchased from Toxin Technology (Sarasota, FL), and Staphylococcal peptidoglycan was obtained from Sigma.

Generation of SEE Mutants

Wild-type SEE lacking the signal peptide was cloned by PCR from *S. aureus* strain FRI913 into a modified version of the pET41a expression plasmid (Novagen) where the enterokinase cleavage site (DDDDK) is replaced with the tobacco etch virus (TEV) protease cleavage site (ENLYFQG). The SEE Y205A and K148A mutants were generated by megaprimer PCR. Proteins were expressed from *E. coli* BL21(DE3) and purified by Ni^{2+}-column chromatography (McCormick and Schlievert, 2003), and the purification tags were removed with auto-inactivation-resistant His_7::TEV (Kapust et al., 2001). The purified proteins ran as homogenous, single bands on SDS-PAGE.

Cell Stimulation, Immunoprecipitation, and Western Blotting

Jurkat T cells were stimulated with LG2 cells as antigen-presenting cells (APC) (5:1 ratio) preincubated with SEE for 40 min at 37ºC or with anti-TCR antibody-coated plates. Plate bound anti-TCR wells were prepared by incubation of C305 culture supernatants (1:10 dilution in PBS) at 4ºC followed by extensive washing with PBS. Cells were subsequently lysed in 1% Triton X-100 lysis buffer, and the lysates were directly analyzed by Western blot or after immunoprecipitation with antibodies on protein A/G agarose beads (Chau et al., 1998; Slifka and Whitton, 2001). In the indicated experiments, Jurkat cells or PBMC were preincubated with pertussis toxin or the PLCβ inhibitor U-73122 or its inactive analog U-73343 for 1 hr prior to T cell activation.

Mixed Lymphocyte Reaction

PBMC (1×10^5) from a healthy donor were stimulated with irradiated-PBMC (1×10^5) from a different donor in the presence or absence of 10 μg/ml of anti-CD4 of OKT4 or RPA-T4. After 4 days, supernatants were collected and assessed for IL-2 by ELISA.

IL-2 Production

T cell blasts (5×10^4) were stimulated with MD-DC (1.6×10^3) and SEE. Jurkat T cells (5×10^4) were stimulated with LG2 cells (2×10^4) and SEE in the presence or absence of the indicated protein inhibitors, with plate bound anti-TCR and soluble anti-CD28. For Pertussis toxin experiments, Jurkat cells were incubated with the toxin for 1 hr before adding LG2 and SEE. Cell culture supernatants were collected after 18–24 hr and assayed for IL-2 content by ELISA via OptEIA human IL-2 Set (BD Biosciences). Data were analyzed with MPM III 1.34 (BioRad) and GraphPad Prism software (GraphPad Software Inc.).

Lck Kinase Assay

Jurkat T cells were stimulated with LG2 cells (5:1 ratio) preincubated with SEE for 40 min at 37ºC for 10 min. Cells were subsequently lysed in 1% Triton X-100 lysis buffer, and Lck was immunoprecipitated with antibodies on protein A/G agarose beads (Chau et al., 1998; Slifka and Whitton, 2001). Lck immunoprecipitates were washed twice in the corresponding lysis buffer. Washed Lck immunoprecipitates were then incubated with 30 μl of kinase buffer containing 20 mM Tris (pH 7.6), 10 mM $MnCl_2$, 1 μM ATP, 160 μg/ml rabbit muscle enolase, and 5 μCi [γ^{32}P]ATP for 10 min at room temperature. After incubation, 10 μl of 4× reduced sample buffer was added. Lck immunoprecipitates were boiled for 5 min, centrifugated, and resolved on 8% SDS-PAGE.

Intracellular Free Ca^{2+} Measurements

Jurkat T cells were loaded with the calcium indicator dyes fluo-3AM (4 μg/ml) and Fura Red (10 μg/ml) (Molecular Probes, Inc., Eugene, OR) in Hank's balanced salt solution containing 4 mM Probenecid (Sigma-Aldrich), 1% FBS, 1 mM calcium chloride, and 1 mM magnesium chloride for 30 min at 37ºC. Cells were analyzed in a FACS Calibur cytometer (BD Biosciences). Baseline measurements were collected for 2 min, ionomycin (2 μg/ml), SEE-pulsed LG2 cells, or unpulsed LG2 cells were added, and measurement was continued after pelleting Jurkat cells and LG2 cells, for an additional 15 min period. Stimulus-induced changes in the intracellular Ca^{2+} concentration were determined over time by monitoring the fluorescence emission ratio fluo-3AM/Fura Red. Data were analyzed and plots were generated with FlowJo software (Tree Star, Inc.).

Analysis of CD69 Upregulation by Flow Cytometry

Jurkat T cells were stimulated in 6-well plates with soluble anti-CD3 antibodies, with anti-CD3 coated beads, or with LG2 cells and SEE at the indicated concentrations for 16 hr, harvested, and stained with FITC-labeled anti-CD69. Cells were examined by flow cytometry with a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA).

Nuclear and Cytoplasmic Fractions Extraction

Jurkat T cells were stimulated with LG2 cells and SEE as described above. Next, nuclear and cytoplamic fractions were prepared with the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL), according to manufacturer's recommendations. Nuclear and cytoplamic fractions were resolved by SDS-PAGE and analyzed by Western blotting. Purity of the cytoplasmic and nuclear fractions was tested by immunoblotting for ERK-1 and ERK-2 and histone H2A, respectively.

RNA Interference for Gα11, PLC-β_1, and Lck Gene Silencing in JCaM1.6 Cells

The small interfering RNA (siRNA) against *Gα11* was obtained from Qiagen (Germantown, MD). The antisense sequence of the selected oligos to target Gα11 gene were r(AUG GAC UCC AGA GUC AUC G)dTdC and r(UUG AGG AAG AGG AUG ACG G)dAdG. siRNA against PLC-β_1 was purchased from Ambion (Austin, TX). The antisense sequence of the selected oligos to target PLC-β_1 gene were AAGUCUAUUAACUUUUGGGtc, UUUUGAGAGGAAUACGCCCtt, and AUGGGAGAUGUUCACGAGGtc. siRNA against Lck was performed with the si*GENOME SMART*pool reagent purchased from Dharmacon (Lafayette, CO). Transfection was done with the Nucleofector kit for cell lines from Amaxa biosystems (Gaithersberg, MD) according to the manufacturer's protocol. After 24 hr, siRNA

or control transfected cells were harvested and processed for cell lysate preparation and IL-2 production.

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