Glucocorticoid-mediated Inhibition of Lck Modulates the Pattern of T Cell Receptor-induced Calcium Signals by Down-regulating Inositol 1,4,5-Trisphosphate Receptors

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Glucocorticoids are potent immunosuppressive agents that block upstream signaling events required for T cell receptor (TCR) activation. However, the mechanism by which glucocorticoids inhibit downstream responses, such as inositol 1,4,5-trisphosphate (IP₃)-induced calcium signals, is not completely understood. Here we demonstrate that low concentrations of dexamethasone rapidly convert transient calcium elevations to oscillations after strong TCR stimulation. Dexamethasone converted the pattern of calcium signaling by inhibiting the Src family kinase Lck, which was shown to interact with and positively regulate Type I IP₃ receptor. In addition, low concentrations of dexamethasone were sufficient to inhibit calcium oscillations and interleukin-2 mRNA after weak TCR stimulation. Together, these findings indicate that by inhibiting Lck and subsequently down-regulating IP₃ receptors, glucocorticoids suppress immune responses by weakening the strength of the TCR signal.

Glucocorticoids are among the most widely prescribed immunosuppressive agents, due in part to their remarkable ability to inhibit synthesis of pro-inflammatory cytokines such as IL-2. Typically, glucocorticoid hormones function to stimulate the activation and nuclear translocation of the glucocorticoid receptor, a ligand-activated transcription factor that trans-activates or represses genes that regulate cell proliferation and apoptosis. In T cells, the ligand-bound glucocorticoid receptor represses synthesis of IL-2 by interfering with transcription factors that regulate cytokine gene expression. More recently, glucocorticoids have been shown to rapidly inhibit upstream mediators of TCR signaling by a non-genomic mechanism that does not require nuclear translocation of the ligand-bound glucocorticoid receptor. Although it is likely that both of these mechanisms work cooperatively to suppress TCR activation, it is uncertain how rapid effects of glucocorticoids affect downstream responses, such as inositol 1,4,5 trisphosphate (IP₃)-induced calcium signals.

Calcium is a versatile second messenger that is necessary for the activation and proliferation of T lymphocytes. TCR stimulation induces calcium release from the ER to the cytosol by way of IP₃ receptor channels. This process is mediated, in part, by the Src family kinase Lck, which is abundantly expressed in immature double positive T cells. Lck localizes to the cell surface after antigenic stimulation and is activated by tyrosine autophosphorylation. After its activation, Lck phosphorylates downstream effector molecules leading to the activation of phospholipase Cγ, which in turn catalyzes the hydrolysis of phosphatidylinositol 4,5 bisphosphate, thereby generating IP₃ and inducing calcium mobilization. Moreover, sustained cytosolic calcium elevation stimulates cytokine gene expression through the activation of calcineurin and nuclear translocation of NFAT.

The strength of TCR activation determines the magnitude of calcium responses. For example, antigenic peptides that induce strong TCR activation generate higher concentrations of cytosolic calcium relative to peptides that induce weak activation. Furthermore, stimulation with high concentrations of the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) inhibitor thapsigargin or anti-CD3 antibody generates transient calcium elevations, whereas low concentrations induce oscillations. In agreement with these findings, we demonstrated that strong TCR stimulation induced by a high concentration of anti-CD3 generates a single transient calcium elevation that peaks 1–2 min after the addition of antibody. In contrast, weak TCR stimulation, induced by a lower concentration of anti-CD3, generates sustained calcium oscillations. In the present study we found that 30–60 min of exposure to low concentrations of dexamethasone converted calcium signaling patterns from transient to oscillatory after strong TCR stimulation and inhibited oscillations induced by weak TCR stimulation. Because it was previously shown that Src kinase activity is inhibited by dexamethasone, we hypothesized that inhibition of Lck might be responsible for conversion of the calcium signaling pattern. After targeting Lck with the Src kinase inhibitor dasatinib and specifically knock-down its expression with siRNAs, we determined that this modulation in calcium signaling was dependent on Lck. More-
Glucocorticoids Modulate Calcium Signals

over, calcium responses were mediated in part by a protein-protein interaction between Lck and Type I IP₃ receptor, and loss of Lck expression or activity resulted in IP₃ receptor down-regulation. Together these data suggest that glucocorticoid-mediated inhibition of Lck controls the pattern of TCR responses by negatively regulating IP₃ receptor expression. These data provide a novel mechanism by which glucocorticoids function to inhibit calcium signaling to suppress TCR stimulation.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Fura-2AM was purchased from Invitrogen. Dasatinib (BMS-354825, Sprycel) and thapsigargin were purchased from LC laboratories (Woburn, MA). Dexamethasone was purchased from Sigma. Lck and IP₃ receptor siRNAs were purchased from Dharmacon (Lafayette, CO). The following antibodies were used in this study: anti-mouse CD3ε (BD Biosciences); Lck (3A5) (Santa Cruz Biotechnology, Santa Cruz, CA); phospho-Lck Tyr-394 (Sgc Tyr-416, 2101) (Cell Signaling Technology, Danvers, MA); β-actin (AC-15) (Sigma). Antibody against Type I IP₃ receptor was kindly provided by Jan Parys and Humbert De Smedt (KU, Leuven, Belgium) (26). Antibodies for Type I and Type II IP₃ receptors were also kindly provided by Richard Wojcikiewicz (CT-1 and CT-2; SUNY, Syracuse, NY) (27, 28).

Cell Culture—WEHI7.2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, L-glutamine (2 mM), and nonessential amino acids (100 µM).

Western Blotting—For all Western blotting experiments, cells were washed in phosphate-buffered saline and lysed in cold SDS sample buffer. After denaturation, lysates were quantified by the Bradford assay and subjected to SDS-PAGE using a 4–15% gradient gel. Proteins were transferred to a polyvinylidene difluoride membrane and probed with the appropriate antibody and incubated overnight at 4 °C. Immunocomplexes were visualized by exposure to chemiluminescent substrates. Protein levels were normalized to β-actin and quantified by densitometry.

Single Cell Calcium Imaging—Cells were plated at a concentration of 7.5 × 10⁵ cells/ml on poly-l-lysine-coated coverslips (MatTek, Ashland, MA). Cells were allowed to adhere to coverslips for ~1 h by incubating at 37 °C. After incubation, cells were loaded with 1 µM fura-2AM for 45 min at 25 °C. Cell culture media was then replaced with CaBSS buffer (pH 7.5) containing NaCl (130 mM), KCl (5 mM), CaCl₂ (1.5 mM), MgCl₂ (1 mM), Heps free acid (25 mM), glucose (5 mM), and bovine serum albumin (1 mg/ml) and incubated for 30 min at 25 °C to allow for de-esterification. Cells were visualized using an inverted Zeiss axiovert S100 microscope and 20× Fluor objective (Carl Zeiss AG, Oberkochen, Germany). Excitation filters were alternated between 340 and 380 nm using a filter wheel with exposure times of ~1 s for each wavelength. The output was filtered at 510 nm, collected with a charge-coupled device camera (Hamamatsu Photonics, Shizuoka, Japan), and converted to a digital image using PCI software for Windows (Hamamatsu Photonics). To stimulate cells, anti-CD3 antibody was slowly added to the coverslip ~1 min after establishing a basal calcium measurement. Thapsigargin-releasable calcium was measured after the addition of thapsigargin (1 µM) and EGTA (10 mM) to chelate extracellular calcium. In these experiments, the difference between the peak and basal calcium level was calculated to obtain the Δ calcium value. For all experiments, calcium concentration was determined empirically using Rₘₐₓ and Rₘᵢₙ values and 220 nm as the Kₙ for fura-2 (29). Rₘᵢₙ values were obtained by stimulating calcium release with ionomycin (10 µM) while chelating extracellular calcium with EGTA (4 mM). Rₘₐₓ values were obtained by stimulating calcium release with ionomycin (10 µM) while perfusing cells with CaCl₂ (4 mM). For further details, see Zhong et al. (25).

Transfection of siRNAs—Before transfection, WEHI7.2 cells were resuspended at a concentration of 5 × 10⁵ cells/ml in serum-free media (Invitrogen). 1 × 10⁵ cells were mixed with non-targeting or Lck siRNA (SMARTpool) oligonucleotides (Dharmacon) at a concentration of 1000 nM in a 0.20-cm cuvette. Cells were electroporated with a single 140-V, 10-ms² wave pulse (Bio-Rad) and transferred into fresh serum-containing media. Cells were incubated under standard cell culture conditions for 24 h before being assayed. Three individual SMARTpool siRNAs specifically targeting each IP₃ receptor subtype were transfected into WEHI7.2 cells as described above. Non-targeting siRNAs were transfected at a concentration of 3000 nM to control for each individual IP₃ receptor siRNA (1000 nM). Cells were incubated under standard cell culture conditions for 48 h before being assayed.

Immunoprecipitation—In immunoprecipitation experiments, 5 × 10⁷ to 1 × 10⁸ cells were cultured at 2–5 × 10⁶ cells/ml depending on the time-course of the assay. Cells were washed twice in phosphate-buffered saline, and pellets were resuspended in buffer containing Tris (50 mM), NaCl (100 mM), EDTA (2 mM), CHAPS (1%), NaF (50 mM), Na₃VO₄ (1 mM), phenylmethylsulfonyl fluoride (1 mM) and protease and phosphatase inhibitor cocktails (Roche Applied Science). Total protein (0.5–4 mg) was immunoprecipitated with the appropriate antibody and incubated overnight at 4 °C. Immune complexes were incubated with protein G-agarose beads (Millipore, Billerica, MA), washed in phosphate-buffered saline/lysis buffer, denatured and boiled in SDS sample buffer, and subjected to Western blotting. In coimmunoprecipitation experiments with peptides, WEHI7.2 lysates were prepared as above. Total protein (4 mg) was incubated with peptides (200 µM) for 1 h at 4 °C before immunoprecipitation with the appropriate antibody.

GST Pulldown Assays—pGEX6p2 plasmids containing N-terminal GST-tagged IP₃ receptor fragments were kindly provided by Jan Parys and Humbert De Smedt (KU, Leuven, Belgium). Fragments were expressed in bacterial cells and purified as previously described (30, 31). Lysates from WEHI7.2 cells were prepared in radioligand precipitation assay buffer containing Tris-HCl (50 mM), NaCl (150 mM), EDTA (1 mM), Nonidet P-40 (1%), sodium deoxycholate (0.5%), NaF (1 mM), Na₃VO₄ (1 mM), and protease inhibitor mixture (Roche Applied Science). Purified GST-IP₃ receptor fragments (10–20 µg) were mixed with 25 µl of Sepharose beads and incubated with 300 µg of total protein for 1 h at 4 °C. To minimize non-specific binding, the NaCl concentration was increased to 300 mM, and 1% bovine serum albumin was added to the mixture. GST-IP₃ receptor complexes were washed in radioligand
precipitation assay buffer, eluted in glutathione buffer, and subjected to Western blotting. Purified GST-IP$_3$ receptor fragments were subjected to SDS-PAGE, and bands were visualized by staining with Coomassie Brilliant Blue.

**Peptide Synthesis and Delivery**—Peptides were synthesized by Genscript (Piscataway, NJ), and purity was $\geq 95\%$ as documented by mass spectrometry and high pressure liquid chromatography. The control peptide is a 20-amino acid-scrambled sequence derived from murine Type I IP$_3$ receptor. The sequence of the control peptide is NLNHSDQFAE-NLSHICGGHG. The domain 5-derived peptide is a 21-amino acid peptide 100% homologous to amino acids 2078–2098 of murine Type I IP$_3$ receptor. The sequence of the domain 5-derived peptide is KKR-MDLVLELKNASKLLLAI. Peptides were delivered into WEHI7.2 cells using Chariot reagent (Active Motif, Carlsbad, CA). Peptides (20 or 200 $\mu$M) were incubated with prediluted Chariot regent for 30 min at 37 °C. Peptide and Chariot complexes were added to cells (weakly adhered to poly-L-lysine-coated coverslips) in serum-free media for 30 min and replenished with 1 ml of complete media. Cells were then prepared for calcium measurements, as described.

**Real-time Quantitative PCR**—WEHI7.2 cells were plated at a concentration of $5 \times 10^5$ cells/ml, preincubated with 0.1% ethanol or 10 and 100 nM dexamethasone for 4 h, and stimulated with anti-CD3 (2 $\mu$g/ml). Cells were incubated with dexamethasone for an additional 16 h before being lysed in Trizol (Invitrogen). RNA was isolated by conventional phenol/chloroform methods, precipitated in isopropanol, washed in 75% ethanol, resuspended in RNase-free water, and quantified by measuring the optical density at 260/280 nm. 4 $\mu$g of total RNA was reverse-transcribed using the RETROscript kit (Ambion, Austin, TX). 1 $\mu$l of cDNA was combined with the appropriate volume of PCR master mix, which was then transferred to tubes containing IL-2 TaqMan reagents (Applied Biosystems, Foster City, CA). PCR reactions were amplified in a 7500 fast real-time PCR thermal-cycler (Applied Biosystems). Each sample was quantified by the $\Delta\Delta$Ct method using $\beta$-actin as the endogenous control.

**Cell Viability**—WEHI7.2 cells were plated at a concentration of $5 \times 10^5$ cells/ml and left untreated or incubated with dexamethasone (1 and 10 nm) for 60 min or 0.01% DMSO or dasatinib (100 nM) for 30 min. Cells were washed in phosphate-buffered saline and resuspended in buffer containing HEPES (10
mM), NaCl (140 mM), and CaCl$_2$ (2.5 mM). Cells were incubated with propidium iodide (1 μg/ml) for 15 min and analyzed by flow cytometry using an EPICS XL-MCL flow cytometer (Beckman Coulter, Fullerton, CA).

**Statistical Analysis**—Student’s t test was used to determine statistical significance between two groups. Two tailed p values are shown where appropriate. All statistics were generated by Microsoft Excel 2004 for Macintosh.

**RESULTS**

**Strong and Weak TCR Stimulation Induce Distinct Patterns of Calcium Signaling**—Immature T cells are highly sensitive to calcium responses induced by anti-TCR antibodies. In this study we have utilized WEHI7.2 cells because they are (i) double positive (i.e. expresses cell surface antigens CD4 and CD8), (ii) similar to cortical thymocytes, (iii) undergo robust calcium responses that can be manipulated by the concentration of TCR agonist, and (iv) sensitive to the effects of glucocorticoids. With the use of single cell digital imaging, calcium responses were monitored in real time after stimulation with anti-CD3. Strong TCR stimulation with a relatively high concentration of anti-CD3 (13–20 μg/ml) generated single transient calcium elevations (Fig. 1, A and B, left). Transient elevations were broad in shape, high in amplitude, and occurred within 1–2 min after stimulation. On the other hand, weak TCR stimulation with a lower concentration of anti-CD3 (2 μg/ml) generated calcium...
oscillations that were low in amplitude, high in frequency, and persisted for up to 30 min after stimulation (Fig. 1, A and B, right). Note that Fig. 1C illustrates the importance of measuring calcium responses at a single cell level. Because of the irregularity and lower amplitude of calcium oscillations generated by weak TCR stimulation, an average trace (n/1500566 cells) shows relatively no response compared with the average trace of transient elevations that are more synchronous with faster response kinetics. Thus, the single cell traces in Fig. 1, A and B, more accurately depict the pattern of calcium responses than is possible in methods that measure average calcium in a cell population.

**Glucocorticoids Regulate the Pattern of Calcium Responses after Strong TCR Stimulation**—Glucocorticoids rapidly inhibit TCR signaling; however, it is not known whether they affect specific patterns of calcium responses. In an effort to better understand how glucocorticoids affect TCR-induced calcium signals, we treated WEHI7.2 cells with low concentrations of dexamethasone (1–10 nM) for relatively short periods of time to ensure that cell viability was not compromised during these experiments. We found that pretreatment with 1–10 nM dexamethasone for 30–60 min was sufficient to convert the calcium signaling pattern from transient to oscillatory (Fig. 2, A–C and E). The effect of dexamethasone in these experiments was strikingly similar to the data in Fig. 1 where weak stimulation induced sustained calcium oscillations, suggesting that glucocorticoids decrease the strength of TCR signaling. In addition, the frequency of calcium oscillations was elevated in cells treated with dexamethasone for 30–60 min compared with cells treated for 4 h (Fig. 2, C and D), implying that glucocorticoids weaken TCR-induced calcium responses in a time-dependent manner. Overall, 30–60 min of dexamethasone treatment increased the frequency of calcium oscillations relative to control cells by greater than 4-fold (Fig. 2E). Moreover, 10 nM dexamethasone also decreased TCR responses in peripheral blood T cells by converting transient elevations to oscillatory signals, indicating that this phenomenon also occurs in normal primary T lymphocytes (supplemental Fig. 1).

In glucocorticoid-sensitive T cells, such as WEHI7.2, prolonged exposure to higher concentrations of dexamethasone (100–1000 nM) results in apoptosis as well as increased levels of cytosolic calcium, which may confound responses to anti-CD3 stimulation. Importantly, 1–10 nM dexamethasone had no effect on the pool of thapsigargin-releasable calcium in
WEHI7.2 cells (Fig. 2F). When cell viability was assessed by propidium iodide staining and flow cytometry, there was no difference between control and dexamethasone-treated cells. Untreated (control) cells were 96% (±0.05%) viable compared with cells treated with 1 and 10 nM dexamethasone, which were 97% (±0.14%) and 96% viable (±0.18%), respectively. These results indicate that low concentrations of glucocorticoids are sufficient to inhibit TCR-induced calcium responses without affecting internal calcium stores and are not a consequence of cell death.

Inhibition of Lck Converts the Pattern of Calcium Responses after Strong TCR Stimulation—Lck is a Src family kinase that is abundantly expressed in double-positive T cells, indispensable for generating TCR-induced calcium responses, and rapidly inhibited by glucocorticoids (9, 32). Thus, it is reasonable to hypothesize that glucocorticoids function to weaken TCR-induced calcium responses by inhibiting Lck. Here we found that low concentrations of dexamethasone reduced Lck phosphorylation at the activating tyrosine residue, Tyr-394, which is required for autophosphorylation and downstream activity (Fig. 3, A–C). In resting cells, Lck phosphorylation was reduced after prolonged treatment with dexamethasone (e.g. 24 h) (Fig. 3A), whereas in stimulated cells phosphorylation of Lck was rapidly inhibited within 60 min (Fig. 3, B and C). To better target Lck, we employed the Src kinase inhibitor dasatinib, which suppresses the activity of Src family kinases in the low nanomolar range (33). Pretreatment with 100 nM dasatinib for 30 min inhibited Lck (Tyr-394) phosphorylation in stimulated cells (Fig. 3D) and converted transient calcium elevations to oscillatory signals in a similar fashion as dexamethasone (Fig. 3, E–G). Dasatinib also caused significant latency in each cell's response to TCR stimulation due to the fact that oscillations were not observed until 15–20 min after the addition of anti-CD3 (Fig. 3, E and F). Dasatinib also had no effect on thapsigargin-releasable calcium (Fig. 3H). Cell viability, as assessed by propidium iodide staining, was 97% (±0.52%) and 97%

FIGURE 4. siRNA-mediated knockdown of Lck converts the pattern of calcium responses after strong TCR stimulation. WEHI7.2 cells were transfected with siRNA oligonucleotides targeting Lck (or non-targeting control siRNAs) as described under “Experimental Procedures.” Cells were incubated for 24 h before single cell digital imaging and Western blotting. A, shown is a Western blot for Lck to confirm knockdown. NT, non-targeting. B, seven representative single cell calcium traces of non-targeting control or Lck knockdown cells are shown. Cells were stimulated with 13 μg/ml anti-CD3 1 min after t0. C, three additional traces are shown representing the calcium signaling pattern of a single cell (note that the y axes differ to better visualize the oscillations). D, an average determination of spikes/cell for non-targeting control (n = 4) or Lck knockdown cells (n = 4) from four independent experiments is shown. E, shown are the average Δcalcium measurement (i.e. [TG-induced calcium peak] − [resting calcium]) of non-targeting control or Lck knockdown cells after stimulation with thapsigargin (1 μM) as a measure of ER calcium. Error bars represent the S.E.
The finding that rapid treatment with dexamethasone or dasatinib converts the pattern of TCR-induced calcium responses suggests that Lck (or other Src family members) may be responsible for regulating these calcium signals. To directly test this hypothesis, we transiently knocked-down Lck expression in WEHI7.2 cells and measured calcium responses after strong TCR stimulation. siRNA-mediated knockdown of Lck reduced protein levels by nearly 80% (Fig. 4A). Like dexamethasone and dasatinib, a reduction in Lck expression also converted the pattern of calcium signaling from transient to oscillatory after strong TCR stimulation (Fig. 4, B–D). The conversion of calcium responses in Lck knockdown cells was not because of a loss in the ER-releasable calcium pool, as assessed by stimulation with thapsigargin (Fig. 4E). Collectively, these data indicate that glucocorticoid-mediated inhibition of Lck converts the pattern of responses after strong TCR stimulation.

siRNA-mediated Knockdown of Lck Inhibits Calcium Release after Stimulation with IP$_3$ Ester—To further elucidate the mechanism by which Lck regulates the pattern of calcium responses after strong TCR stimulation, we stimulated cells with IP$_3$ ester to bypass the TCR signaling pathway. Because Lck functions in part to mediate production of IP$_3$, we reasoned that calcium responses would be restored in Lck knockdown cells after stimulation with a high concentration (25 μM) of cell permeant IP$_3$ ester (p-myo-IP$_3$ hexakisbutyryloxymethyl ester) which should act directly on IP$_3$ receptors. Surprisingly, calcium responses to IP$_3$ ester were markedly inhibited when Lck was knocked down by >75% (Fig. 5, A–C), suggesting that Lck also regulates calcium responses downstream of IP$_3$ production. Importantly, only 11% of Lck knockdown cells showed a ≥2-fold response to IP$_3$ ester compared with 51% in control cells. Furthermore, in the population of responding cells, there was increased latency and slower kinetics in Lck knockdown cells. The latency period after the addition of IP$_3$ ester for control and Lck knockdown cells was 1.9 and 4.8 min, respectively.

Lck Interacts with Type I IP$_3$ Receptor to Regulate Patterns of Calcium Signaling—Because IP$_3$ ester did not restore calcium responses in Lck knockdown cells, we reasoned that inhibition of Lck may have altered IP$_3$ receptor expression or function. We discovered that Lck coimmunoprecipitated with Type I IP$_3$ receptor, indicating that these two proteins are in complex with one another (Fig. 6A). To more closely examine the mechanism of the Lck-IP$_3$ receptor interaction, we performed pulldown experiments using GST-tagged fragments of murine Type I IP$_3$ receptor. Each fragment comprises domains 1–6, which are shown schematically in Fig. 6B. After incubating WEHI7.2 lysate with each IP$_3$ receptor fragment, we determined that Lck interacted at domain 5, which is composed of 285 amino acids (1932–2216) and is part of the regulatory and coupling domain of Type I IP$_3$ receptor (Fig. 6C).

Next, we assessed whether the Lck-IP$_3$ receptor interaction was necessary for mediating calcium responses after strong TCR stimulation. To test this, we set out to design an IP$_3$ receptor-derived peptide that would function as a competitive inhibitor.
to displace Lck. We used bioinformatic approaches to predict the region of domain 5 that was likely to interact with Lck. Specifically, we used the GORIV method to target a region of domain 5 that was predicted to form \( \beta \)-helicity (34). An additional algorithm, developed by Rost and Sander (35) and Rost et al. (36), was used to validate these target sequences. Based on these predictions, we chose a 21-amino acid region in the middle of domain 5 (amino acids 2078–2098) beginning with two lysine residues that were also strongly predicted to facilitate protein-protein interactions (36, 37). The sequence of the 21-amino acid \( \text{IP}_3 \) receptor domain 5-derived peptide is shown in Fig. 6D. As a control, we designed and synthesized a scrambled peptide, also from Type I \( \text{IP}_3 \) receptor, that had no significant homology to any mammalian protein.

To determine whether this peptide was capable of inhibiting the Lck-\( \text{IP}_3 \) receptor interaction, we incubated WEHI7.2 lysate with either control or domain 5-derived peptide for 1 h before immunoprecipitation with Type I \( \text{IP}_3 \) receptor or IgG antibody.
The domain 5-derived peptide successfully inhibited the coimmunoprecipitation, suggesting that the region of domain 5 comprising amino acids 2078–2098 of Type 1 IP₃ receptor was responsible for interacting with Lck (Fig. 6E). Importantly, delivery of the domain 5-derived peptide into WEHI7.2 cells via Chariot reagent converted transient calcium elevations to oscillations after strong TCR stimulation, further demonstrating that this interaction is necessary for mediating the appropriate calcium response (Fig. 6F–H).

**Figure 7.** Down-regulation of IP₃ receptors converts the pattern of calcium responses after strong TCR stimulation. A, cells were transfected with siRNA oligonucleotides targeting Lck (or non-targeting control siRNAs). Cells were lysed 24 h post-transfection and subjected to Western blotting for Lck and Type I IP₃ receptor (IP₃R). NT, non-targeting. B, cells were preincubated with 0.1% ethanol or 1 nM dexamethasone (Dex) for 60 min and stimulated with anti-CD3 for 5 min. Lysates were subjected to Western blotting for Type I IP₃ receptor. C, cells were preincubated with dasatinib (Das) for 60 min to inhibit Lck activity, Lysates were subjected to Western blotting for Type I IP₃ receptor. Ctrl, control. D, WEHI7.2 cells were transfected with siRNA oligonucleotides targeting each IP₃ receptor subtype (or non-targeting control siRNAs). Cells were incubated for 48 h before single cell digital imaging. A Western blot for IP₃ receptors to confirm knockdown of each subtype is shown. E, seven representative single cell calcium traces of non-targeting control or IP₃ receptor triple knockdown cells are shown. Cells were stimulated with 20 μg/ml anti-CD3 1 min after t₀. F, three additional traces are shown representing the calcium signaling pattern of a single cell (note that the y axes differ to better visualize the oscillations). G, an average determination of spikes/cell for non-targeting control (n = 4) or IP₃ receptor triple knockdown cells (n = 4) is shown. Results are representative of multiple independent experiments. Error bars represent the S.E.

**Down-regulation of IP₃ Receptors Converts the Pattern of Calcium Responses after Strong TCR Stimulation—**Because Lck interacts with Type I IP₃ receptor to regulate calcium signaling, we reason that the loss or inhibition of Lck would not only perturb this interaction but might also affect IP₃ receptor expression. In support of this hypothesis, we observed that Type I IP₃ receptor was profoundly down-regulated in Lck knockdown cells (Fig. 7A). Moreover, dexamethasone, which inhibited Lck phosphorylation, decreased expression of Type I IP₃ receptor within 60 min of treatment in both resting and stimulated WEHI7.2 cells (Fig. 7B). Similar results were obtained when cells were incubated in the presence of 10–100 nM dasatinib (Fig. 7C), suggesting that Lck is required to maintain IP₃ receptor expression.

To assess whether this down-regulation of IP₃ receptor expression was responsible for the conversion of the calcium signaling pattern, we knocked-down IP₃ receptor expression using siRNAs that targeted each receptor subtype. We successfully knocked-down Type I IP₃ receptor by greater than 50% and Types II and III by greater than 90% (Fig. 7D). Consistent with our previous observations from dexamethasone-treated cells, transient calcium elevations were converted to oscillations after strong TCR stimulation (Fig. 7E–G). Notably, calcium oscillations and spikes were observed in these cells within 4 min after the addition of anti-CD3. Thus, knockdown of IP₃ receptors also converts the pattern of calcium signals, indicating that the reduction of Type I IP₃ receptor in the presence of dexamethasone is partly responsible for weakening the TCR signaling pathway.
Lck Regulates Calcium Oscillations Induced by Weak TCR Activation

Because Lck regulated transient calcium elevations after strong TCR activation, we asked whether it would also affect those oscillations and spikes, which are normally induced by weak TCR activation (Fig. 1). After weak TCR stimulation with a lower concentration of anti-CD3, calcium oscillations were decreased in cells treated with 1–10 nM dexamethasone (Fig. 8A). The higher concentration of dexamethasone significantly delayed calcium responses given that oscillations were not observed until 15 min after stimulation. To be sure that this mechanism was Lck-dependent, we treated cells with dasatinib to inhibit its activity and knocked down its expression with siRNAs (see Fig. 4A for documentation of knockdown). Dasatinib markedly inhibited the response to weak TCR stimulation (Fig. 8B), and similarly, a decrease in Lck expression reduced the total number of calcium oscillations and increased the latency period after the addition of anti-CD3 (Fig. 8C). Thus, Lck is not only required for the generation of calcium transients after strong TCR stimulation but is also required for oscillations induced by weak stimulation.

Because a reduction in sustained calcium signaling would also inhibit downstream responses, we measured IL-2 levels after TCR stimulation in the presence of dexamethasone. As expected, dexamethasone prevented the induction of IL-2 mRNA that resulted from weak TCR stimulation (Fig. 9). Collectively, these data support the hypothesis that glucocorticoids function to modulate immune responses through the inhibition of Lck and subsequent down-regulation of Type I IP₃ receptor.

**FIGURE 8. Glucocorticoids inhibit calcium responses after weak TCR stimulation by an Lck-dependent mechanism.** A, WEHI7.2 cells were preincubated with 1 or 10 nM dexamethasone for 60 min. Cells were stimulated with 2 μg/ml anti-CD3 1 min after t₀, and seven representative single cell calcium traces are shown for control and dexamethasone-treated cells. Results are representative of two independent experiments. Cntrl, control. B, WEHI7.2 cells were preincubated with 0.01% DMSO or 100 nM dasatinib for 30 min to inhibit Lck activity. Cells were stimulated with 2 μg/ml anti-CD3 for 1 min after t₀, and seven representative single cell calcium traces are shown for control and dasatinib treated cells. Results are representative of two independent experiments. C, WEHI7.2 cells were transfected with siRNA oligonucleotides targeting Lck (or non-targeting control siRNAs). Cells were incubated for 24 h before single cell digital imaging and Western blotting (see Fig. 4A for documentation of Lck knockdown). Cells were stimulated with 2 μg/ml anti-CD3 for 1 min after t₀, and seven representative single cell calcium traces are shown for non-targeting control or Lck knockdown cells. Results are representative of three independent experiments. A low percentage of ethanol (0.001%) was used as a vehicle for dexamethasone and had no effect on calcium responses.

**DISCUSSION**

Glucocorticoids are strong inhibitors of TCR activation and signaling. However, the mechanism by which glucocorticoids rapidly exert these effects has not been fully investigated. Here we report that the synthetic glucocorticoid dexamethasone rapidly diminishes TCR responses by modulating the pattern of calcium signaling. In the case of strong stimulation, dexamethasone converted calcium responses from transient elevations to sustained oscillations. Additionally, calcium oscillations induced by weak TCR

Lck Regulates Calcium Oscillations Induced by Weak TCR Stimulation—Because Lck regulated transient calcium elevations after strong TCR activation, we asked whether it would also affect those oscillations and spikes, which are normally
Glucocorticoids Modulate Calcium Signals

FIGURE 9. Glucocorticoids inhibit IL-2 synthesis after weak TCR stimulation. WEHI7.2 cells were preincubated with 10 or 100 nm dexamethasone (Dex) for 4 h and stimulated with 2 µg/ml anti-CD3 for 16 h. RNA was isolated from cells and reverse-transcribed into cDNA, and IL-2 mRNA was quantified by real-time PCR. Results represent the mean ΔΔCt value from triplicate measurements using β-actin as the endogenous control. Error bars represent the S.E. Cntrl, control.

stimulation were decreased by dexamethasone, which was associated with the inhibition of IL-2 induction. Importantly, because of the short term exposure and low concentration of dexamethasone used in these experiments, the effects of glucocorticoids reported here were not due to a loss in cell viability or depleted stores of ER calcium that occurs after prolonged glucocorticoid treatment (38, 39). Thus, these results carry important therapeutic implications given that low concentrations of dexamethasone are sufficient to inhibit TCR responses without causing cytotoxic effects on glucocorticoid-sensitive T cells.

It is widely recognized that glucocorticoids suppress immune responses by inhibiting cytokine gene transcription as well as upstream kinases that regulate TCR signaling. This is mediated through the inhibition of AP-1 and/or NFAT activity (6–8, 40), most likely by a mechanism involving protein-protein interactions with the ligand-bound glucocorticoid receptor (1). Glucocorticoids have also been shown to inhibit the transcriptional activation of NFκB (41, 42) as well as up-regulate expression of IκB, thereby preventing transcription of proinflammatory cytokines (43). In addition, glucocorticoids inhibit upstream TCR signaling by redistributing Src proteins and decreasing phosphorylation of ZAP-70 but do so by a mechanism that requires long term exposure to dexamethasone and de novo protein synthesis (44). Although each of these events unequivocally contribute to the immunosuppressive function of glucocorticoids, they do not occur for hours or even days because of the time needed for ligand-bound glucocorticoid receptors to mediate changes in gene expression (10).

More recently, non-genomic effects of glucocorticoids have been described where biological responses are observed within minutes (45). Here we show that immature T cells respond to 1 nm dexamethasone within 30–60 min by converting the pattern of calcium signaling from transient to oscillatory. Rapid effects on calcium signaling induced by glucocorticoids have been previously reported in other cell types. For example, glucocorticoids rapidly stimulate calcium uptake in synaptic membranes after depolarization (46), whereas other studies determined that dexamethasone inhibited calcium transients in neuroendocrine cells within minutes (47, 48). Moreover, in T cells, glucocorticoids block TCR signaling within 30 min by inhibiting Src kinase activity and causing their dissociation from the TCR machinery (9, 11). Our data provide evidence that the rapid conversion in calcium signaling induced by glucocorticoids is because of the inhibition of Lck. For example, when Lck was knocked down by siRNAs or its activity was inhibited by dasatinib, the calcium signaling pattern was modulated in a similar manner as with dexamethasone treatment.

To address how glucocorticoid-mediated inhibition of Lck modulates calcium signals, we stimulated cells with a high concentration of IP₃ ester to bypass the TCR signaling pathway. Although it is possible that inhibition of Lck decreased endogenous levels of IP₃, stimulation with IP₃ ester was not sufficient to restore calcium responses, indicating that Lck deficiency abrogates this pathway downstream of IP₃ production. This led us to hypothesize that inhibition of Lck by glucocorticoids may have perturbed IP₃ receptor expression or function. In a previous study, angiotensin, a vasoconstrictor that increases corticosteroid production, was shown to down-regulate IP₃ receptors in liver cells (49). Our results were similar in that glucocorticoids down-regulated IP₃ receptors via Lck inhibition. This regulation of IP₃ receptors is further supported by evidence that Lck binds to domain 5 of Type I IP₃ receptor, and disruption of this interaction also attenuates TCR-induced calcium responses. Other studies have shown that the Src homolog Fyn interacts with and phosphorylates Type I IP₃ receptor to enhance TCR-induced calcium release (50–53). Thus, Fyn and Lck may both function to regulate the pattern of TCR-induced calcium responses by interacting with IP₃ receptors. Although Fyn is expressed at considerably lower levels in double positive T cells (14) and in WEHI7.2 cells, siRNA-mediated knockdown of Fyn also inhibited strong TCR stimulation (data not shown).

It is important to note that long term exposure (16–24 h) to high concentrations of dexamethasone (100–1000 nm) results in the up-regulation of IP₃ receptors (54). We speculate that this up-regulation in IP₃ receptor expression may be secondary to the immediate effect of glucocorticoid treatment, which is the inhibition of Lck and subsequent down-regulation of IP₃ receptor expression. In this way, prolonged exposure to dexamethasone may up-regulate IP₃ receptors as a form of feedback to compensate for an acute down-regulation in their expression. Alternatively, the up-regulation of IP₃ receptors by glucocorticoids may be induced by a distinct mechanism that requires transcriptional activation. This concept is supported by the observation that IP₃ receptor mRNA levels are also increased after hours of exposure to dexamethasone (54).

In summary, we have shown that glucocorticoids, by inhibiting Lck and down-regulating Type I IP₃ receptors, modulate distinct patterns of calcium responses after both strong and weak TCR stimulation in immature T cells. Perhaps therapies that target Src kinase activity (e.g. dexamethasone or dasatinib) will help to suppress or modulate immune responses while
minimizing cytotoxicity that is typically accompanied with chronic exposure to higher doses of glucocorticoids.

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