A Live Imaging Cell Motility Screen Identifies Prostaglandin E₂ as a T Cell Stop Signal Antagonist

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A Live Imaging Cell Motility Screen Identifies Prostaglandin E2 as a T Cell Stop Signal Antagonist

Andrew J. Wiemer,* Subramanya Hegde,* Jenny E. Gumperz,* and Anna Huttenlocher*†

The T cell migration stop signal is a central step in T cell activation and inflammation; however, its regulatory mechanisms remain largely unknown. Using a live-cell, imaging-based, high-throughput screen, we identified the PG, PGE2, as a T cell stop signal antagonist. Src kinase inhibitors, microtubule inhibitors, and PGE2 prevented the T cell stop signal, and impaired T cell–APC conjugation and T cell proliferation induced by primary human allogeneic dendritic cells. However, Src inhibition, but not PGE2 or microtubule inhibition, impaired TCR-induced ZAP-70 signaling, demonstrating that T cell stop signal antagonists can function either upstream or downstream of proximal TCR signaling. Moreover, we found that PGE2 abrogated TCR-induced activation of the small GTPase Rap1, suggesting that PGE2 may modulate T cell adhesion and stopping through Rap1. These results identify a novel role for PGs in preventing T cell stop signals and limiting T cell activation induced by dendritic cells. *The Journal of Immunology, 2011, 187: 3663–3670.

T cells are highly migratory cells that travel at speeds up to 30 μm/min (1) and during inflammation can arrest their migration in response to receptor-mediated signals (2). T cells receive a migration “stop signal” and can rapidly halt migration after TCR signaling and interaction with APCs. Transient T cell stopping can also be sufficient to induce T cell activation under some conditions (3, 4), and more prolonged interactions can be associated with the generation of CD8+ memory T cells (5). In any case, the T cell stop signal is essential for some types of immune synapse formation and T cell activation (6), and represents an attractive therapeutic target. The molecular mechanisms controlling the T cell stop signal are not well understood but likely involve signaling through one or more of the TCR proximal kinases Lck, Fyn, and ZAP-70, and activation of the small GTPase Rap1 (7). Inhibiting proximal TCR signaling through ligation of the endogenous receptor CTLA-4 can reverse the T cell stop signal (8).

Despite its importance we have limited understanding of the signaling pathways that regulate T cell arrest induced by TCR engagement. In this study, we describe a live-cell, imaging-based, high-throughput method to identify signaling pathways that control the T cell stop signal induced by TCR ligation. Using live imaging, we can assess the kinetics that accompany the decision to stop or go in response to TCR engagement and we can identify small molecules that modify the kinetics of T cell stopping, and thereby may impact duration of T cell–APC interactions. This is especially important in light of recent studies that demonstrate that the duration of T cell–APC contacts can determine T cell fate and the development of T cell activation or tolerance (9).

In this study, we identified PGE2 as a novel regulator of T cell stopping and T cell–dendritic cell (DC) interactions. PGs are bioactive lipids that have been implicated in inflammation and are targeted by cyclooxygenase (COX) inhibitors commonly used to treat inflammatory disease (10, 11). However, the precise mechanisms by which PGs control inflammation are not well understood, and recent studies have suggested that specific PGs, notably PGE2, may have anti-inflammatory effects (12, 13). Our findings provide novel insight into how PGE2 may limit T cell activation by impairing T cell arrest and inhibiting T cell–DC interactions. Moreover, our studies demonstrate that PGE2, unlike Src kinase inhibition, alters T cell stopping downstream of ZAP-70 and linker of activated T cells (LAT) phosphorylation at the level of Rap1 GTPase activation, indicating that the T cell stop signal can be decoupled from proximal TCR signaling.

Materials and Methods

Reagents and supplies

OKT3 Ab was purified from a B cell hybridoma line (14), and ICAM-1–Fc was purified from transfected CHO cells (15) by affinity to protein G-Sepharose. Calcein-acetoxymethyl ester (AM) was obtained from Invitrogen (Carlsbad, CA). FTY720 was from Cayman Chemical (Ann Arbor, MI). PHA, PP2, U-73122, and PGE2 were obtained from Fisher Scientific.

T cell purification

Leukocytes were obtained from whole blood (16) using Lymphoprep and resuspended in fresh T cell media (RPMI 1640, 10% heat-inactivated FBS, 1× HEPES, pyruvate, nonessential amino acids, β-mercaptoethanol). Cells were stimulated with PHA and expanded in the presence of IL-2 (50 U/ml; Chiron) for 5–10 d. For conjugation and proliferation assays, fresh human T cells were magnetically purified by a negative selection method using a pan T cell isolation kit (Milkenyi Biotec, Auburn, CA).

Generation of DCs

DCs were obtained as described previously (17, 18). Monocytes were purified by magnetic sorting with anti-CD14 beads (Miltenyi Biotec) and differentiated 3 d in RPMI 1640 media supplemented with 2 mM...
and negative controls.

Plate coating

The 384-well tissue culture-treated plates (BD Optilux) were coated by addition of ICAM (20 µl to each well) at 5 µg/ml in coating buffer (Tris pH 9.5). Plates were incubated at 37°C for 1 h, washed, and blocked by addition of 50 µl blocking buffer (2% BSA in 1X PBS) for 1 h at 37°C.

Quantification of adhesion

Cells were suspended at 500,000/ml and labeled by addition of 1 µM calcein-AM for 15 min. Cells were collected by centrifugation and washed twice with media. Cells were plated and centrifuged at 500 × g for 5 min, then incubated at 37°C for 30 min. Migration was monitored using a BD Pathway microscope. Two replicates were analyzed by flow cytometry. Adherent cells were quantified by plate reading.

Stop signal assay

We established conditions (modified from Ref. 8) for modeling the T cell stop signal in a high-throughput assay (Supplemental Table I). Human peripheral blood T (HPBT) cells were labeled with calcein-AM (Invitrogen). A total of 50,000 HPBT cells (1 × 10⁶/ml after 5–14 d of expansion) was added to each well of a 384-well plate. Cells were plated and centrifuged at 500 × g for 5 min, then incubated for 5 min at 37°C. For low-throughput experiments, cells were washed twice using pre-equilibrated culture media, and test compounds were added by pipetting. For high-throughput experiments, cells were mixed six times with a robotic pipette and media exchanged once. Test compounds (6–12 µM) were added using a 384-well pin transfer device. The T cell stop signal was induced with soluble OKT3 (1 µg/ml), a CD3 Ab known to stimulate TCR signaling (14), for 10 min after plating and washing the cells. Migration was monitored using a BD Pathway microscope. Two images were acquired under 10× magnification at an interval of ~7.5 min. Cells in the t = 0 image were outlined with a three-pixel dilation width to define the region of interest (ROI) for each cell, and intensity within the ROI was measured. The remaining intensity was subtracted, and the percentage remaining intensity for each individual ROI was calculated according to the equation \( I_{\text{final}} - I_{\text{ROI}} = I_{\text{background}} \), where \( I_{\text{pixel intensity}} \) and \( BG \) is background intensity. ROIs (cells) with \( I_{\text{final}} \) values >0.5 were deemed stopped, whereas those with values <0.5 were migrating. Hits were defined by having a percentage migration >40%. Treatment with OKT3 caused rapid depolarization, stopped migration within minutes, and changed the distribution of the population of T cells from primarily migratory to stopped (Supplemental Video I). Typically, 60–80% of control cells were migrating compared with 5–20% of the OKT3-treated cells. This approach yielded an average Z' factor of 0.5 and a 6.7-fold difference between positive and negative controls.

2 dimensional/OKT3 stop signal

A total of 50 µl HPBT cells (after 5–14 d of expansion) was added to each well of a 384-well plate. Cells were plated and centrifuged at 500 × g for 5 min, then incubated for 5 min at 37°C. Cells were washed twice using pre-equilibrated culture media, and test compounds were added by pipetting. Cells were incubated with compounds for 30 min at 37°C. Stop signal was induced by addition of OKT3 Ab for 10 min at 37°C. Cells were placed on movable microscope stage in a climate-controlled chamber set at 37°C. Images were obtained using Metamorph, and data were analyzed by Microsoft Excel. For typical experiments, images were acquired for 15 min at 30- or 45-s intervals simultaneously for 8–12 experimental conditions. Migration was quantified by cell tracking; typically, 30 cells picked at random per video were tracked.

Cell-based conjugation assay

DCs were stimulated for 8 h with 250 ng/ml LPS (Sigma-Aldrich, St. Louis, MO), DCs were labeled with DiD (Invitrogen). T cells were labeled with 2.5 µM CFSE. T cells (1 × 10⁶ cells in 100 µl) were treated for 30 min with indicated concentration of compounds, then mixed at a 1:1 ratio with DCs in media. Mixture was immediately centrifuged at 800 × g for 3 min and incubated at 37°C for the indicated time. Mixture was vortexed for 30 s and analyzed by flow cytometry.

T cell proliferation assay

T cells were labeled with 2.5 µM CFSE and cultured at a 50:1 ratio with allogenic LPS-matured DCs with inhibitors or DMSO. Proliferation was assessed on day 6 by flow cytometry. The percentage of live T cells that had undergone cell division was determined by gating on DAPI-CD3+ cells and assessing the fraction that showed diminished CFSE fluorescence intensity.

Western blot analysis

T cells (days 5–10) at 2 × 10⁶/ml in 0.5–1 ml media were incubated with compounds for 30 min. Cells were placed on ice for 5 min, then coated with 1 µg/ml OKT3 on ice for 20 min. Cells were suspended in 100 µl media containing goat anti-mouse F(ab')₂, at 37°C for 3 min. Cells were lysed with 500 µl ice-cold radioimmunoprecipitation assay buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS). Lysing buffer contained freshly added phosphatase inhibitor mixture (1:100 dilution, P-5726; Sigma) and protease inhibitor mixture (1:100 dilution, P-8340; Sigma). Proteins were resolved by SDS-PAGE on 10% gels, transferred to nitrocellulose, and blotted with p-ZAP-70-Y319 (Cell Signaling), p-LAT-Y191 (Cell Signaling), p-Lck-Y505 (Cell Signaling), p-Y (4G10), vinculin (Sigma), p-Src-416 (Cell Signaling), p-Src-529 (Cell Signaling), or Fyn (Santa Cruz) Ab. Detection was performed using Alexa Fluor 680 goat anti-mouse IgG (Molecular Probes) and IRDye 800CW goat anti-rabbit IgG (Rockland).

Rap1 activation assay

Activated HPBT cells (25 × 10⁶) were suspended in 1 ml fresh T cell media in the presence or absence of test compounds for 30 min at 37°C and stimulated with OKT3 as described earlier. Cells were lysed in 500 µl Rap1 lysis buffer (1% Triton X-100; 50 mM Tris-HCl, pH 7.5; 150 mM NaCl, 10 mM MgCl₂; 1 mM PMSF; 1 mM leupeptin; 0.5 mM aprotinin (19). Lysates were cleared by centrifugation (16,000 rpm for 10 min) and incubated with glutathione S-transferase-RalGDS-Rap-binding domain (Millipore) for 1 h at 4°C with rotation. Beads were washed three times with lysis buffer and subjected to Western blot analysis with anti-Rap1 (Santa Cruz). Twenty-five microliters lysate was reserved to use as a loading control.

IL-2 production

Activated HPBT cells (1.5 × 10⁶) in 1 ml T cell media were added to 24-well plates that had been precoated for 2 h with 1 µg/ml OKT3 in PBS. Cells were stimulated for 5 h at 37°C in the presence of brefeldin A (eBioscience). Cells were pelleted by centrifugation and resuspended in FACS buffer (2% FBS in PBS). Cells were labeled with allophycocyanin-CD4 (eBioscience) according to manufacturer’s protocol, washed, and fixed overnight with 4% paraformaldehyde. Cells were resuspended in FACS buffer containing 0.1% saponin for 15 min. Cells were blocked for 15 min with Fc block (eBioscience) and labeled for 30 min with FITC-IL-2 (eBioscience). Cells were washed twice with FACS buffer and analyzed by flow cytometry.

Statistical analysis

All columns in bar graphs represent the mean of the indicated number of replicates. Error bars on graphs represent SEMs. ANOVA with Tukey’s post hoc testing was used to calculate statistical significance. Unless otherwise indicated, comparisons were done relative to the control. An a level of 0.05 was used as the level of significance.

Results

The Src inhibitor, PP2, is a T cell stop signal antagonist

To identify novel regulators of the T cell migration stop signal, we developed an image-based, high-throughput screen for small molecules that impair TCR-induced T cell arrest (Fig. 1, Supplemental Table I, Supplemental Video I). The T cell stop signal was induced in primary human T cells by treatment with the anti-CD3 Ab OKT3 as described in Materials and Methods (Fig. 1A). Soluble OKT3 was sufficient to induce the T cell stop signal without TCR cross-linking or costimulation with anti-CD28 Ab, which is generally required to induce full T cell activation and proliferation, suggesting that early TCR engagement may be sufficient to induce the T cell stop signal (Fig. 1B, Supplemental Video 1).

Lck and Fyn both play a critical role in proximal TCR signal transduction (20). To determine whether inhibition of proximal TCR signal transduction was sufficient to block the stop signal, we pretreated T cells with the Src kinase inhibitor PP2, which inhibits both Lck and Fyn activity (20). PP2 had no effect on T cell
random motility on ICAM-1 but blocked T cell arrest induced by TCR ligation with OKT3 (Fig. 1B). In the presence of both OKT3 and PP2, primary human T cells on ICAM-1 demonstrated rapid, random motility comparable with untreated control T cells on ICAM-1. The findings demonstrate that Src kinases are required for the T cell stop signal but not T cell random motility.

**Src kinase inhibitors PP1 and SU6656, but not SKI-1, impair TCR-induced T cell arrest**

To determine whether blocking proximal TCR signal transduction using other Src family kinase inhibitors was also sufficient to block the TCR-mediated T cell stop signal, we used a panel of other Src inhibitors including PP1, SU6656, or SKI-1 (21). PP1 and SU6656, like PP2, impaired TCR-induced T cell arrest (Fig. 1C). Pretreatment with PP1, SU6656, or SKI-1 had no effect on T cell random motility on ICAM-1. However, SU6656, but not SKI-1, blocked the TCR stop signal and impaired adhesion of T cells to ICAM-1-coated plates (Fig. 1D). Accordingly, we also found that SU6656, but not SKI-1, inhibited phosphorylation of ZAP-70 and LAT under the conditions of our assay (Fig. 1E,1F). Taken together, we found that the three different Src kinase inhibitors that impaired proximal TCR signaling also blocked the TCR-mediated T cell stop signal.

**PGE2 impairs TCR-induced T cell arrest**

To identify novel signaling pathways involved in T cell stop signals, we screened a library of 1600 compounds for effects on OKT3-induced T cell arrest as described in Materials and Methods and Supplemental Table I (Fig. 2, Supplemental Table II). Cells were plated on ICAM-1–coated 384-well plates in the presence or
absence of test compounds. Approximately 60% of control cells (green line) were actively migrating, whereas stimulation with OKT3 induced a stop signal, decreasing migration to ∼10% (Fig. 2A, red line). The majority of test compounds did not alter the ability of T cells to stop migrating (Fig. 2A, gray dots). After confirmation, five compounds, 0.31% of the total screened, prevented the T cell stop signal (Fig. 2A). The positive compounds included two PGs (PGE₁ and PGE₂) and three microtubule-disrupting compounds (colchicine, albendazole, and nocodazole; Table I). Representative time-lapse images for PP2, PGE₂, and colchicine are shown in Fig. 2B. Control cells that were actively migrating do not colocalize at times \( t = 0 \) and \( t = 7.5 \), whereas cells stopped with OKT3 colocalized. Treatment with PP2, PGE₂, or colchicine was sufficient to reverse colocalization and block T cell stopping.

To confirm these results, we performed time-lapse microscopy to track the kinetics of T cell motility in T cells stimulated with OKT3 in the presence and absence of test compounds (Fig. 3). As previously reported, primary human T cells were highly polarized and motile on ICAM-1–coated plates (Fig. 3A). Treatment with OKT3 induced a loss of T cell polarity and impaired migration (from 10 μm/min in control T cells to 3 μm/min with OKT3; Fig. 3). The effects of OKT3 on cell polarity and T cell motility were prevented by either treatment with PGE₂ or the microtubule-disrupting compound, colchicine (Fig. 3B, 3C, Supplemental Videos 2–4). The other positive hits also increased T cell polarity and random motility in the presence of OKT3 (Supplemental Fig. 1A); however, PP2 was most effective at reversing T cell stopping. Taken together, these results identify both PGs (PGE₂) and microtubule-disrupting agents (colchicine) as novel T cell stop signal antagonists. The identification of PGs as stop

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signal antagonists is particularly surprising because PGs have previously been reported to inhibit T cell-directed migration (22).

PP2, but not PGE2 or colchicine, impairs proximal TCR signal transduction

Src kinase inhibition with PP2 prevents proximal TCR signal transduction and activation of ZAP-70. To determine whether PGE2 functions upstream of proximal T cell signaling to affect T cell arrest, we characterized the effects of PGE2 on phosphorylation of downstream targets (Fig. 4). In accordance with previous reports, stimulation of human T cells with OKT3 for 3 min induced an increase in total tyrosine phosphorylation, which was blocked by PP2 but was not altered by treatment with PGE2 or colchicine. To determine whether PGE2 affects ZAP-70 phosphorylation, we examined phosphorylation on tyrosine 319 (23). OKT3 strongly stimulated ZAP-70 Y319 phosphorylation, which was prevented by treatment with PP2. In contrast, PGE2 and colchicine, at concentrations that abrogate T cell arrest, had no effect on OKT3-induced ZAP-70 phosphorylation. PP2, but not PGE2 or colchicine, prevented OKT3-induced ZAP-70 activation and phosphorylation of LAT at Y191. Moreover, PP2, but not PGE2 or colchicine, decreased levels of the activating Fyn phosphorylation at Y420. Taken together, these results demonstrate that PGE2 and colchicine, in contrast with PP2, function as T cell stop signal antagonists downstream or independently of proximal T cell signal transduction. These results were surprising because PGE2 has been reported to inhibit Src kinase activity under some conditions (10, 24).

PGE2 impairs TCR-induced, LFA-1–mediated adhesion to ICAM-1

TCR engagement induces LFA-1 activation and T cell adhesion to ICAM-1 (25). To determine whether PGE2 modulates TCR-mediated LFA-1 activation, we tested the effects of PGE2 on T cell adhesion to ICAM-1 in the presence of OKT3. Treatment with OKT3 induced a 3- to 6-fold increase in adhesion to ICAM-1 relative to untreated control cells (Fig. 5A). PP2 blocked TCR-induced adhesion of T cells to ICAM-1. PGE2 and colchicine also impaired T cell adhesion to ICAM-1 in the presence of OKT3. These results suggest that PGE2 limits T cell arrest by impairing TCR-mediated inside-out LFA-1 activation and adhesion to ICAM-1.

PGE2 impairs TCR-induced Rap1 activation

TCR engagement induces activation of the small GTPase Rap1, which is required for inside out activation of LFA-1 and subsequent adhesion to ICAM-1 (19). To determine whether PGE2 modulates Rap1 activation, we tested the effects of PGE2 on Rap1 GTP binding after stimulation with OKT3. OKT3 induced an ~8-fold increase in GTP-bound Rap1 relative to untreated control cells (Fig. 5B). Both PP2 and PGE2 blocked Rap1 activation (Fig. 5C). These results suggest that PGE2 may limit T cell arrest by pre-
venting Rap1 GTPase activation and impairing LFA-1–mediated adhesion to ICAM-1.

**PGE2 impairs T cell–DC interactions and DC-induced T cell proliferation**

Duration of T cell–DC interactions modulate T cell fate and activation (9). To determine whether PGE2 modulates human T cell–DC interactions, we characterized the effects of PGE2 on the interactions between T cells and allogeneic DCs using flow cytometry. DCs were derived from peripheral blood monocytes treated with GM-CSF and IL-4 for 3 d, followed by LPS for 8 h. Interactions with naive human T cells labeled with CFSE were performed using flow cytometry as described in Materials and Methods (17) (Fig. 6, Supplemental Fig. 1B). In the presence of vehicle control, the efficiency of T cell–DC conjugation was 22.3% (Fig. 6A). PP2 (50 μM) reduced the rate of conjugation to 9.5%, which was reported as TCR-dependent conjugation. Treatment of T cells with PGE2 (50 μM) also inhibited conjugation, with an approximate 60% decrease in TCR-dependent conjugation (Fig. 6B, 6C). To determine whether PGE2 modulates downstream T cell signaling, we tested the effects of PGE2 on TCR-stimulated IL-2 production (Fig. 6D). OKT3 induced a ∼15-fold increase in IL-2 production relative to untreated control cells. PP2, PGE2, and colchicine blocked TCR-induced IL-2 production.

To determine whether PGE2 modulates DC-induced T cell proliferation, we analyzed proliferation of T cells using CFSE dilution at day 6 after stimulation with allogeneic DCs (Fig. 6E, Supplemental Fig. 1C). In the presence of DMSO only, 31% of cells were proliferative. PP2 and PGE2 blocked T cell proliferation at concentrations of 50 μM (0.6 and 0.5% of control, respectively). The inhibition by PGE2 was dose dependent (Fig. 6F), and a statistically significant decrease in proliferation was seen at concentrations as low as 2.5 μM. Growth inhibition was also observed on stimulation with CD3/CD28-coated beads in the presence of PGE2 (Supplemental Fig. 1D). Taken together, these data demonstrate that PGE2 impairs T cell–DC conjugation and DC-induced T cell proliferation.

**Discussion**

The identification of small molecules that alter T cell interactions with APCs represents an intriguing therapeutic strategy for autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE). Indeed, a recent study has highlighted the critical importance of T cell and APC contact duration in determining T cell fate in vivo and the development of T cell tolerance or activation (9). There are currently no known small molecules that reverse the T cell stop signal in clinical use, and the addition of such drugs to treat autoimmune diseases is particularly attractive given the high cost of biologic agents and the resultant burden on the healthcare system. In this study, we have identified at least three distinct classes of “reverse-stop” small molecules that impair TCR-induced T cell arrest but not random T cell motility: 1) Src family tyrosine kinase inhibitors, 2) microtubule depolymerizing agents, and 3) PGs. These compounds act in contrast with inhibitors of phospholipase C (U 73122), which block both basal and activated T cell motility or sphingosine-1-phosphate analog FTY720 and the PI3K inhibitor LY-294002, which altered basal motility but did not affect adhesion or spreading induced by OKT3 (Supplemental Table III).
The requirement of Src family kinases for the TCR-induced T cell stop signal, but not for T cell random motility, indicates that Src inhibitors represent T cell stop signal antagonists. This is consistent with the model that proximal T cell signaling is necessary for TCR-induced T cell arrest. It is intriguing that not all Src kinase inhibitors, most notably SKI, are capable of reversing the T cell stop signal, but not for T cell random motility, indicating that Src inhibitors may act independently of its effects on Src kinase activity. Our findings suggest that it is possible to decouple proximal TCR signaling from the TCR stop signal. PGE2 had no effect on the phosphorylation of Lck or Fyn at concentrations that block T cell arrest, suggesting that PGE2 effects on T cell arrest are independent of its effects on Src kinase activity. Our findings identified a novel role for PGE2 in the regulation of the small GTPase Rap1, which is critical for TCR-induced inside-out activation of LFA-1.

To our knowledge, this is the first report to implicate PGE2 in regulating the T cell stop signal. In contrast, previous studies have reported that PGE2 stimulates the ability of DCs to induce T cell proliferation (30). The finding that PGE1 and PGE2 impair T cell migration stopping, as well as inhibit T cell proliferation (31), indicate there may be counteracting mechanisms in place. Therefore, the presence of PGs may both promote and block DC-dependent T cell activation depending on the context of exposure. In addition, although PGE2 has been largely thought to be proinflammatory, recent studies have suggested that PGE2 and PG analogs may be anti-inflammatory in cases of autoimmune diseases such as SLE (13), because of its effects on DC-mediated cytokine production and shifting immune response from a Th1 to Th2 profile. Inhibition of the TCR stop signal would provide an additional anti-inflammatory mechanism for PGE2.

Interactions between DCs and T cells play a central role in the pathogenesis of autoimmune diseases such as SLE and represent an important therapeutic target. In addition to affecting the TCR stop signal, we found that PGE2 significantly impaired T cell–DC interactions and DC-induced T cell proliferation (Fig. 4). PGE2 and certain PG analogs are Food and Drug Administration-approved agents, and the novel effects on T cell stop signal and interactions with DCs suggest they may have therapeutic benefit in patients with SLE. In support of this possibility is a recent article that suggests that PGE2 also inhibits IFN-α secretion by plasmacytoid DCs, key players in SLE pathogenesis (13). In addition, another report recently demonstrated that COX inhibitors disrupt resolution of inflammation that was dependent on PGE2 in a mouse arthritis model (12). Moreover, our results may help to explain why exacerbation of SLE-like symptoms has been reported in patients treated with COX inhibitors (13), which function to decrease PG synthesis.

In summary, we have identified small molecules that modulate the T cell stop signal using a novel image-based, high-throughput screen. Because the approach is activation based rather than inhibition based, there are likely to be fewer off-target hits. We have

Previous work has indicated that Src kinase activation is required for TCR-mediated polarization of the microtubule-organizing center toward the T cell–APC contact (26) (27). It is interesting that our data indicate that Src inhibitors and microtubule disruption impair T cell stopping and interactions with APC. This is, to our knowledge, the first report to show that microtubules are necessary for the T cell arrest induced by TCR ligation. In accordance with our findings, previous studies have reported that microtubule disruption induces random motility of neutrophils (28) and modulates T cell random migration through rho/ROCK signaling (29). However, ROCK inhibition did not affect TCR-induced T cell stopping in our system, suggesting that effects of microtubule inhibition on T cell arrest may be independent of Rho/ROCK signaling.

The finding that both microtubule polymerization inhibitors and PGs are capable of preventing the T cell stop signal without affecting ZAP-70 or LAT phosphorylation (i.e., proximal TCR signaling) is particularly interesting (Fig. 3). In fact, we had initially hypothesized that the screening results would yield molecules that work to disrupt proximal signaling, such as the Src inhibitors. Our findings suggest that it is possible to decouple proximal TCR signaling from the TCR stop signal. PGE2 had no effect on the phosphorylation of Lck or Fyn at concentrations that block T cell arrest, suggesting that PGE2 effects on T cell arrest are independent of its effects on Src kinase activity. Our findings identified a novel role for PGE2 in the regulation of the small GTPase Rap1, which is critical for TCR-induced inside-out activation of LFA-1.

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The requirement of Src family kinases for the TCR-induced T cell stop signal, but not for T cell random motility, indicates that Src inhibitors represent T cell stop signal antagonists. This is consistent with the model that proximal T cell signaling is necessary for TCR-induced T cell arrest. It is intriguing that not all Src kinase inhibitors, most notably SKI, are capable of reversing the T cell stop signal. The results suggest that the stop signal is dependent on an Src family kinase, which is preferentially targeted by PP1, PP2, and SU6656, but not SKI.

Previous work has indicated that Src kinase activation is required for TCR-mediated polarization of the microtubule-organizing center toward the T cell–APC contact (26) (27). It is interesting that our data indicate that Src inhibitors and microtubule disruption impair T cell stopping and interactions with APC. This is, to our knowledge, the first report to show that microtubules are necessary for the T cell arrest induced by TCR ligation. In accordance with our findings, previous studies have reported that microtubule disruption induces random motility of neutrophils (28) and modulates T cell random migration through rho/ROCK signaling (29). However, ROCK inhibition did not affect TCR-induced T cell stopping in our system, suggesting that effects of microtubule inhibition on T cell arrest may be independent of Rho/ROCK signaling.

The finding that both microtubule polymerization inhibitors and PGs are capable of preventing the T cell stop signal without affecting ZAP-70 or LAT phosphorylation (i.e., proximal TCR signaling) is particularly interesting (Fig. 3). In fact, we had initially hypothesized that the screening results would yield molecules that work to disrupt proximal signaling, such as the Src inhibitors. Our findings suggest that it is possible to decouple proximal TCR signaling from the TCR stop signal. PGE2 had no effect on the phosphorylation of Lck or Fyn at concentrations that block T cell arrest, suggesting that PGE2 effects on T cell arrest are independent of its effects on Src kinase activity. Our findings identified a novel role for PGE2 in the regulation of the small GTPase Rap1, which is critical for TCR-induced inside-out activation of LFA-1.

To our knowledge, this is the first report to implicate PGE2 in regulating the T cell stop signal. In contrast, previous studies have reported that PGE2 stimulates the ability of DCs to induce T cell proliferation (30). The finding that PGE1 and PGE2 impair T cell migration stopping, as well as inhibit T cell proliferation (31), indicate there may be counteracting mechanisms in place. Therefore, the presence of PGs may both promote and block DC-dependent T cell activation depending on the context of exposure. In addition, although PGE2 has been largely thought to be proinflammatory, recent studies have suggested that PGE2 and PG analogs may be anti-inflammatory in cases of autoimmune diseases such as SLE (13), because of its effects on DC-mediated cytokine production and shifting immune response from a Th1 to Th2 profile. Inhibition of the TCR stop signal would provide an additional anti-inflammatory mechanism for PGE2.

Interactions between DCs and T cells play a central role in the pathogenesis of autoimmune diseases such as SLE and represent an important therapeutic target. In addition to affecting the TCR stop signal, we found that PGE2 significantly impaired T cell–DC interactions and DC-induced T cell proliferation (Fig. 4). PGE2 and certain PG analogs are Food and Drug Administration-approved agents, and the novel effects on T cell stop signal and interactions with DCs suggest they may have therapeutic benefit in patients with SLE. In support of this possibility is a recent article that suggests that PGE2 also inhibits IFN-α secretion by plasmacytoid DCs, key players in SLE pathogenesis (13). In addition, another report recently demonstrated that COX inhibitors disrupt resolution of inflammation that was dependent on PGE2 in a mouse arthritis model (12). Moreover, our results may help to explain why exacerbation of SLE-like symptoms has been reported in patients treated with COX inhibitors (13), which function to decrease PG synthesis.

In summary, we have identified small molecules that modulate the T cell stop signal using a novel image-based, high-throughput screen. Because the approach is activation based rather than inhibition based, there are likely to be fewer off-target hits. We have
shown that Src kinase inhibitors potently block the T cell stop signal and impair T cell–DC interactions. Our findings suggest that compounds that function either downstream or independently of ZAP-70 and LAT are also capable of reversing the T cell stop signal. The ability of PGs to block TCR-induced Rap1 activation and antagonize the T cell stop signal is especially intriguing and supports the use of this class of compounds as therapeutic agents that may have benefit in autoimmune disease. Likewise, these results may help explain the surprising proinflammatory effects sometimes seen with COX-2 inhibitors. Taken together, the findings suggest that small molecules that reverse the migration stop signal at the level of Rap1, or directly induce random motility, thereby limiting TCR-induced stopping and DC-induced T cell activation. This study illustrates that high-throughput imaging of primary human cells can effectively be used to identify small molecules that alter migration stopping, allowing for further understanding of the molecular mechanisms that regulate Ag-induced T cell arrest, and offering a new paradigm for drug discovery.

Acknowledgments

We thank Noel Peters and Song Guo at the University of Wisconsin Keck Small Molecule Screening Facility and Kathy Schell at the University of Wisconsin Flow Cytometry Facility for technical assistance. We thank Sarah Wernimont, Miriam Shelef, David Bennin, and Taylor Starnes for phlebotomy assistance.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Table 1. Outline of assay protocol.

<table>
<thead>
<tr>
<th>Step</th>
<th>Parameter</th>
<th>Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Label cells</td>
<td>1 μM</td>
<td>Label cells in bulk at 1M/mL with calcein-AM</td>
</tr>
<tr>
<td>2</td>
<td>Plate cells</td>
<td>50 μL</td>
<td>20,000 HBPT cells/well</td>
</tr>
<tr>
<td>3</td>
<td>Wash cells</td>
<td>2x</td>
<td>Remove 30 μL of media with 3 mixes at flow rate of 75 μL/s</td>
</tr>
<tr>
<td>4</td>
<td>Controls</td>
<td>5 μL</td>
<td>Final concentration of 5 μM PP2</td>
</tr>
<tr>
<td>5</td>
<td>Library</td>
<td>3 μL</td>
<td>Final concentration of ~30 μM</td>
</tr>
<tr>
<td>6</td>
<td>Incubation time</td>
<td>30 min</td>
<td>37ºC and 5% CO₂</td>
</tr>
<tr>
<td>7</td>
<td>Stop signal</td>
<td>5 μL</td>
<td>Final concentration of 1 μg/μL OKT3</td>
</tr>
<tr>
<td>8</td>
<td>Readout</td>
<td>490 nm ex/520 nm em</td>
<td>BD Pathway imaging system</td>
</tr>
</tbody>
</table>

**Step Notes**

1. Cells labeled for 15 min in incubator and washed 1x with media at 1200 rpm
2. Black BD Optilux 384 well plates plated by Biomek Fx
3. Washed with Biomek Fx
4. Columns 1 and 2, no OKT, Columns 23 and 24, +OKT3. Columns 1, 2, 23, and 24 rows 4, 8, 12, and 16, +PP2.
5. Pin transfer device to columns 3-22
6. Plates kept in dark in standard tissue culture incubator
7. Addition of OKT3 was performed with Biotek micro fill dispenser
8. BD pathway images acquired at 7.5 min intervals. ROIs identified with BD Attovision software using polygon object identification with 3pt pixel dilation width.
Supplemental Table 2. Plate statistics†

<table>
<thead>
<tr>
<th>Plate</th>
<th>Compounds</th>
<th>Initial Migration Hits</th>
<th>Visual Hits</th>
<th>Lab Validated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prestwick Chemical Library Plate 1</td>
<td>320</td>
<td>8</td>
<td>2</td>
<td>2, nocadazole, albendazole</td>
</tr>
<tr>
<td>Prestwick Chemical Library Plate 2</td>
<td>320</td>
<td>12</td>
<td>1</td>
<td>1, PGE2</td>
</tr>
<tr>
<td>Prestwick Chemical Library Plate 3</td>
<td>320</td>
<td>11</td>
<td>2</td>
<td>1, colchicine</td>
</tr>
<tr>
<td>NIH Clinical Collection Plate 1</td>
<td>320</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NIH Clinical Collection Plate 2</td>
<td>320</td>
<td>4</td>
<td>3</td>
<td>1, PGE1</td>
</tr>
<tr>
<td><strong>AVERAGE/TOTAL</strong></td>
<td><strong>1600</strong></td>
<td><strong>45 (2.8%)</strong></td>
<td><strong>9 (0.5%)</strong></td>
<td><strong>5 (0.31%)</strong></td>
</tr>
</tbody>
</table>

†All screening performed at the University of Wisconsin Keck Small Molecule Screening Facility.
Supplemental Table 3. Miscellaneous migration targeted compounds.

<table>
<thead>
<tr>
<th>Treatment (mechanism)</th>
<th>Effect on Migration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control OKT3</td>
<td>no effect</td>
</tr>
<tr>
<td>FTY720 (S1P)</td>
<td>round/detach no effect</td>
</tr>
<tr>
<td>LY-294002 (PI3K)</td>
<td>round/detach no effect</td>
</tr>
<tr>
<td>U73122 (PLC)</td>
<td>round/attached round/attached</td>
</tr>
<tr>
<td>EGTA (divalent ion chelator)</td>
<td>round/detach round/detach</td>
</tr>
<tr>
<td>Latrunculin (actin)</td>
<td>round/detach round/detach</td>
</tr>
<tr>
<td>Cytoclasin D (actin)</td>
<td>round/detach round/detach</td>
</tr>
<tr>
<td>Y-27632 (ROCK)</td>
<td>tail retraction tail retraction</td>
</tr>
<tr>
<td>ALLM (calpain)</td>
<td>no effect</td>
</tr>
<tr>
<td>NSC23766 (rac)</td>
<td>no effect</td>
</tr>
<tr>
<td>CP-339818 (Kv1.3)</td>
<td>no effect</td>
</tr>
<tr>
<td>Aspirin (COX-1/2)</td>
<td>no effect</td>
</tr>
<tr>
<td>Ibufrofin (COX-1/2)</td>
<td>no effect</td>
</tr>
<tr>
<td>SP-600125 (JNK)</td>
<td>Not tested no effect</td>
</tr>
<tr>
<td>PD-98059 (MEK)</td>
<td>Not tested no effect</td>
</tr>
<tr>
<td>U-0126 (MEK)</td>
<td>Not tested no effect</td>
</tr>
<tr>
<td>SB203580 (P38)</td>
<td>Not tested no effect</td>
</tr>
<tr>
<td>BpV (PTEN)</td>
<td>Not tested no effect</td>
</tr>
</tbody>
</table>
**Supplemental Figure 1.** A) Reversal of T cell migration stop signal by microtubule depolymerizing agents and prostaglandins. Cell velocity (μm/min) of HPBT cells migrating on ICAM-1 in the presence or absence of colchicine, nocodozole, albendazole, PGE1 or PGE2 (all at 5 μM) and OKT3 (1 μg/mL) during a 15 minute period. Hits were verified by time-lapse DIC microscopy as described in Materials and Methods and their migration speeds expressed as mean +/- SEM (n=3). B) PP2 and PGE2 inhibit T cell-DC conjugation. CFSE-labeled T cells were mixed with allogeneic DiD-labeled DCs and assessed for conjugation in the presence or absence of PP2 (50 μM), colchicine (50 μM) or PGE2 (50 μM) for either 0 or 30 minutes. Conjugation events as a percentage of total events for one representative experiment is indicated in boxed area. C) PGE2 inhibits DC-induced T cell proliferation. CFSE-labeled T cells were activated by DCs and analyzed for proliferation by CFSE dilution at 6 days post-stimulation in the presence or absence of PGE2, colchicine or PP2 (all at 2.5, 10 and 50 μM). Data is representative from three independent experiments. D) PP2, PGE2, and colchicine inhibit proliferation of T cells stimulated with CD3/CD28 coated beads. 50,000 T cells at day 6-10 post-isolation were labeled with CFSE and stimulated with an equivalent amount of beads for 3 days in the presence or absence of compounds at indicated concentrations (mean +/- SEM, n=3, p<0.05).

**Supplemental Table 1. Outline of assay protocol.** hPBT cells were isolated and assessed between day 6-10 for migration in the presence or absence of test compounds and/or OKT3 antibody as described in Materials and Methods.
**Supplemental Table 2. Plate statistics.** 1600 compounds from the Prestwick Chemical Library and the NIH Clinical Collection were assessed for ability to prevent the T cell migration stop signal as described in Materials and Methods and Results sections.

**Supplemental Table 3. Miscellaneous migration targeted compounds.** Seventeen additional compounds which alter various aspects of T cell receptor signaling were tested for their ability to alter T cell migration in the presence or absence of OKT3 as described in Materials and Methods.

**Supplemental Movie S1. 2 point time lapse of untreated or OKT3-treated hPBT cells on ICAM-1.** hPBT cells were stained with calcein, loaded onto an ICAM-1 coated 384-well plate, and stimulated with OKT3 as described in Materials and Methods. Cells were imaged at T=0 minutes and T=7.5 minutes using a BD Pathway microscope. Data is representative of three independent experiments.

**Supplemental Movie S2. 15 minute time lapse of hPBT cells alone or treated with OKT3, PP2, or OKT3+PP2.** hPBT cells were loaded onto an ICAM-1 coated 384-well plate, and stimulated with OKT3 in the presence or absence of PP2 (10 μM) as described in Materials and Methods. Cells were imaged for 15 minutes. Movies are representative of three independent experiments.

**Supplemental Movie S3. 15 minute time lapse of hPBT cells alone or treated with OKT3, PGE2, or OKT3+PGE2.** hPBT cells were loaded onto an ICAM-1 coated 384-
well plate, and stimulated with OKT3 in the presence or absence of PGE2 (10 μM) as described in Materials and Methods. Cells were imaged for 15 minutes. Movies are representative of three independent experiments.

Supplemental Movie S4. 15 minute time lapse of hPBT cells alone or treated with OKT3, Colch, or OKT3+Colch. hPBT cells were loaded onto an ICAM-1 coated 384-well plate, and stimulated with OKT3 in the presence or absence of colchicine (10 μM) as described in Materials and Methods. Cells were imaged for 15 minutes. Movies are representative of three independent experiments.