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 Cyman 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 selected by a negative selection method using a pan T cell isolation kit (Miltenyi 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...


**Table I, Supplemental Video 1).** 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 dilution width to define the region of interest (ROI) for each cell, and intensity within the ROI was calculated. The value of pixel intensity was subtracted, and the percentage remaining intensity for each individual ROI was calculated according to the equation 

\[ \text{percentage remaining} = \frac{I_{\text{initial}} - I_{\text{final}}}{I_{\text{initial}}} \times 100 \]

where \( I_{\text{initial}} \) and \( I_{\text{final}} \) represent the mean initial and final intensity for each cell, respectively. The ROI size was set to include the tail of the cell, as defined by the cell outline. Values were set between 0.5 and 6.7-fold difference between positive and negative controls.

**Rap1 activation assay**

Activated HPBT cells (25 × 10^6) 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; 100 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.

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**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 test was used to calculate statistical significance. Unless otherwise indicated, comparisons were done relative to the control. An α 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 1). 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 crosslinking 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-induced 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 compounds. PGE₂ impaired TCR-induced T cell arrest (Fig. 2A). To confirm the effect of PGE₂ on TCR-induced T cell arrest, we used a high-throughput assay (Fig. 2B). Calcein-labeled HPBT cells were plated in 384-well plates and screened as described in Materials and Methods. PGE₂ impaired TCR-induced T cell arrest in a dose-dependent manner (Fig. 2C). PGE₂ treatment disrupted the formation of T cell clusters and impaired T cell adhesion to ICAM-1 (Fig. 2D). Taken together, these findings suggest that PGE₂ impairs TCR-induced T cell arrest.

**FIGURE 1.** Src kinase inhibitors prevent the T cell stop signal. A, Schematic of methods used to distinguish between motile and nonmotile cells. B, The fraction of migratory cells significantly decreased with OKT3 treatment and was prevented by addition of 5 μM PP2 (representative data from >15 experiments, 32 replicates each, mean ± SD). C, Src kinase inhibitors reversed the OKT-3–mediated T cell stop signal. Cells were pretreated with 5 μM Src inhibitors PP1, PP2, SKI-1, and SU6656 (mean ± SD; n = 3; *p < 0.05 by ANOVA). D, SU6656 inhibits T cell adhesion to ICAM-1. Cells were incubated for 30 min with indicated concentrations (mean ± SEM; n = 3; *p < 0.05 by ANOVA). E, PP2 and SU6656, but not SKI-1, inhibited phosphorylation of ZAP-70 and LAT. Cells were incubated for 30 min with compounds and stimulated for 3 min with OKT3 as described in Materials and Methods. Representative blots from three independent experiments are shown. F, Quantification of Western blots (mean ± SEM; n = 3; *p < 0.05, ANOVA with Tukey’s post hoc test).

**FIGURE 2.** A high-throughput assay for the HPBT cell migration stop signal identifies PGE₂ and microtubule inhibitors as T cell stop signal antagonists. A, PGE₁, PGE₂, colchicine, nocodazole, and albendazole impaired TCR-induced T cell stopping. Calcein-labeled HPBT cells (50,000/well) were plated and screened as described in Materials and Methods. B, Representative images at t = 0 and t = 7.5 min from primary screen after 30-min treatment with selected compounds (PP2, PGE₂, and colchicine; ~6–12 μM) and 10-min treatment with OKT3 (1 μg/ml). Merged image shows overlapped cells in the presence of OKT3 alone that is reversed by PP2, PGE₂, and colchicine. Scale bar, 100 μm.
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

Table I. Confirmed hits from screening 1600 compounds of known bioactivity

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<tr>
<td>Dinoprostone (PGE2)</td>
<td></td>
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<td>Microtubules</td>
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<td>Nocodazole</td>
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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).
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 in vitro may either impair proximal TCR signaling, inhibit signaling 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
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Disclosures
The authors have no financial conflicts of interest.

References
### Supplemental Table 1. Outline of assay protocol.

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<tr>
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<td>Controls</td>
<td>5 μL</td>
<td>Final concentration of 5 μM PP2</td>
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<td>Readout</td>
<td>490 nm ex/520 nm em</td>
<td>BD Pathway imaging system</td>
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### 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 †

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<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</td>
<td>OKT3</td>
</tr>
<tr>
<td>FTY720 (S1P)</td>
<td>round/detach</td>
</tr>
<tr>
<td>LY-294002 (PI3K)</td>
<td>round/detach</td>
</tr>
<tr>
<td>U73122 (PLC)</td>
<td>round/attached</td>
</tr>
<tr>
<td>EGTA (divalent ion chelator)</td>
<td>round/detach</td>
</tr>
<tr>
<td>Latrunculin (actin)</td>
<td>round/detach</td>
</tr>
<tr>
<td>Cytoclasin D (actin)</td>
<td>round/detach</td>
</tr>
<tr>
<td>Y-27632 (ROCK)</td>
<td>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>Ibuprofin (COX-1/2)</td>
<td>no effect</td>
</tr>
<tr>
<td>SP-600125 (JNK)</td>
<td>Not tested</td>
</tr>
<tr>
<td>PD-98059 (MEK)</td>
<td>Not tested</td>
</tr>
<tr>
<td>U-0126 (MEK)</td>
<td>Not tested</td>
</tr>
<tr>
<td>SB203580 (P38)</td>
<td>Not tested</td>
</tr>
<tr>
<td>BpV (PTEN)</td>
<td>Not tested</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.