T cells must migrate in order to encounter antigen-presenting cells (APCs) and to execute their varied functions in immune defense and inflammation. ATP release and autocrine signaling through purinergic receptors contribute to T cell activation at the immune synapse that T cells form with APCs. Here, we show that T cells also require ATP release and purinergic signaling for their migration to APCs. We found that the chemokine stromal-derived factor-1α (SDF-1α) triggered mitochondrial ATP production, rapid bursts of ATP release, and increased migration of primary human CD4+ T cells. This process depended on pannexin-1 ATP release channels and autocrine stimulation of P2X4 receptors. SDF-1α stimulation caused localized accumulation of mitochondria with P2X4 receptors near the front of cells, resulting in a feed-forward signaling mechanism that promotes cellular Ca2+ influx and sustains mitochondrial ATP synthesis at levels needed for pseudopod protrusion, T cell polarization, and cell migration. Inhibition of P2X4 receptors blocked the activation and migration of T cells in vitro. In a mouse lung transplant model, P2X4 receptor antagonist treatment prevented the recruitment of T cells into allograft tissue and the rejection of lung transplants. Our findings suggest that P2X4 receptors are therapeutic targets for immunomodulation in transplantation and inflammatory diseases.
Introduction

T cells have important roles in host immune defense and inflammation. These involve the migration of T cells into secondary lymphoid organs where they search for matching antigens that are displayed by antigen-presenting cells (APCs). In lymph nodes, cell migration enables T cells to sequentially interrogate APCs for cognate antigens capable of causing T cell activation (1–3). The mechanisms that regulate T cell migration are only partially understood (4, 5). An in-depth understanding of these mechanisms may lead to the development of novel therapeutic strategies to modulate host immune defenses and to prevent the recruitment of T cells into inflamed tissues that are damaged in the course of allergic and autoimmune diseases such as Crohn’s disease, rheumatoid arthritis, lupus erythematosus, and multiple sclerosis (6, 7). T cells that infiltrate donor tissues following organ transplantation cause cellular rejection, which is a particularly serious and unresolved problem in lung transplantation (8, 9).

A large number of chemokines and their corresponding receptors regulate T cell trafficking into target tissues (10). One of these chemokines is stromal-derived factor-1α (SDF-1α), also known as CXCL12) that binds to the chemokine receptor CXCR4 and contributes to the homing of CD4+ T cells into secondary lymphoid organs and regulates cell migration within lymph nodes (11–13). SDF-1α also causes T cell recruitment to the lungs in allergic airway diseases and into lung allografts (14, 15). Here we show that SDF-1α triggers the activation of mitochondrial ATP production and the release of cellular ATP from naive T cells. It is well known that extracellular ATP and purinergic signaling play important roles in the regulation of immunity and inflammation (16–18). We and others have shown that cellular ATP release and autocrine stimulation of P2X1, P2X4, and P2X7 receptors regulate T cell receptor (TCR) signaling, enhance IL-2 production, and induce the proliferation of T cells (19–21). Here we show that ATP release into the pericellular space is also essential for T cell migration, namely by fueling autocrine signaling via P2X4 receptors that regulate pseudopod protrusion and the migration of T cells in response to SDF-1α. We found that pharmacological targeting of P2X4 receptors prevents T cell migration and the recruitment of T cells into lung allograft tissue in a mouse lung transplant model, suggesting that P2X4 receptor signaling is a therapeutic target to prevent organ rejection.

Results

SDF-1α triggers rapid ATP release and T cell migration. Cell migration is a fundamental feature by which T cells traffic to lymph nodes and other tissues to scan APCs for suitable antigens (4). Over a dozen different chemokine receptors regulate T cell trafficking (10). CXCR4 and its ligand SDF-1α are involved in the recruitment of lymphocytes to lymph nodes and allograft tissues (11, 14, 15). T cell trafficking into transplanted lungs leads to graft rejection, which is an unresolved problem that limits success in lung transplantation (9, 22). A better understanding of the mecha-
nisms that regulate T cell migration may reveal novel therapeutic targets to prevent allograft rejection. We found that SDF-1α stimulation of naive CD4+ T cells induces rapid cell polarization, robust migration, and the release of cellular ATP (Figure 1, A and B; Supplemental Figure 1A; and Supplemental Video 1; supplemental material available online with this article; https://doi.org/10.1172/JCI120972DS1). SDF-1α dose-dependently increased migration speed and the area covered by T cells (Figure 1C). Studies with a novel membrane-anchoring ATP probe, 2-2Zn (23, 24), revealed that SDF-1α triggers a sudden surge of ATP release within seconds of cell stimulation (Figure 1, A and D; and Supplemental Video 2). SDF-1α-induced ATP release was blocked by the gap junction inhibitor carbenoxolone (CBX) and the pannexin-1 (PANX1) channel inhibitor 10-panx1 (Figure 1, D and E; and Supplemental Video 2). Next, we examined the role of PANX1 in the migration of Jurkat cells, a CD4+ T cell line that is often used to study CD4+ T cell activation mechanisms and that reacts similarly to SDF-1α stimulation (Supplemental Figure 1). Silencing of PANX1 channels in Jurkat cells blocked SDF-1α-induced ATP release, polarization, and cell migration (Figure 1F and Supplemental Video 3). These findings and similar recent reports by others demonstrate that ATP release via PANX1 channels is required for the migration of CD4+ T cells in response to SDF-1α (25).

**Mitochondria produce the ATP that regulates T cell migration.** In our previous work, we have shown that mitochondria fuel autocrine purinergic feedback mechanisms that maintain basal functions of resting T cells (26). In activated T cells, a burst of mitochondrial activity promotes T cell receptor signaling at the immune synapse between T cells and APCs (27). Here, we show that mitochondria also regulate cell migration. Inhibition of mitochondria by uncoupling oxidative phosphorylation with carbonyl cyanide m-chlorophenyl hydrazine (CCCP) blocked ATP release and the polarization and migration of T cells in response to SDF-1α (Figure 2, A–C). Blocking ATP release or inhibition of P2 receptor stimulation by treating cells with CBX, suramin, or by removing released ATP with apyrase impaired the polarization and migration of cells in response to SDF-1α (Figure 2, B and C). Thus, SDF-1α-induced mitochondrial ATP production fuels autocrine feedback mechanisms that regulate CD4+ T cell migration. In order to study how SDF-1α elicits ATP production, we used Rhod-2 to assess Ca2+ uptake by mitochondria, which is a prerequisite for mitochondrial ATP synthesis (28). Using live-cell imaging with Rhod-2 and 2-2Zn, we found that mitochondrial Ca2+ uptake and hotspots of ATP release coincided with sites of pseudopod protrusion and membrane remodeling (Figure 2, D and E; and Supplemental Video 4). These findings suggest that SDF-1α triggers mitochondrial activation and localized ATP release that regulates pseudopod protrusion at the front of migrating T cells.

**P2X4 receptors regulate T cell migration.** SDF-1α dose-dependently increased the activation of T cells in peripheral blood mononuclear cell (PBMC) cultures stimulated under conditions that necessitate T cell migration for the ligation of both the TCR and CD28 coreceptor (Figure 3A). Adding SDF-1α to PBMC cultures stimulated with soluble anti-CD3 antibodies in flat-bottom dishes increased the migration speed of T cells, the range the cells covered, and the expression of CD69 that was assessed as an early T cell activation event (Figure 3B and Supplemental Figure 2A). There was a positive correlation between parameters of cell migration (migration speed or range) and cell activation (CD69 expression; Figure 3C and Supplemental Figure 2B). While SDF-1α increased cell migration, it did not alter coupling of T cells with anti-TCR/CD28 antibody-coated beads (Supplemental Figure 2, C and D). This suggests that ATP release in response to SDF-1α and autocrine stimulation of purinergic receptors promotes T cell activation by increasing cell migration, the rate of T cell/APC encounters, and the formation of immune synapses. Of the 19 known mammalian purinergic receptor subtypes, which comprise 4 P1 (adenosine), 7 P2X, and 8 P2Y receptors, all 7 ionotropic P2X receptors and several of the G protein–coupled P2Y receptor subtypes (P2Y2, P2Y4, P2Y11) are capable of recognizing ATP (29–31). Of these ATP receptors, CD4+ T cells express the P2X1, P2X4, P2X7, and P2Y11 receptor subtypes (20, 32). The P2X1, P2X4, and P2X7 subtypes contribute to immune synapse signaling, but the role of these receptors in T cell migration is not known (20). We found that 5-(3-bromophenyl)-1,3-dihydro-2H-benzofuro[3,2-e]-1,4-diazepin-2-1 (5-BDBD), a specific and selective P2X4 receptor antagonist (33–35), impaired T cell migration in response to SDF-1α (Figure 3D). Inhibition of P2X1 or P2X7 receptors with NF023 and A438079, respectively, had less effect on cell migration. Inhibition of P2X4 receptors was also more effective than inhibition of P2X1 or P2X7 receptors in suppressing CD69 expression and the proliferation of CD4+ T cells (Figure 3, E and F; and Supplemental Figure 3). Taken together, these findings show that P2X4 receptor signaling has a special role in the regulation of T cell migration.

**P2X4 receptors modulate cytosolic and mitochondrial Ca2+ levels in response to SDF-1α.** P2X4 receptors act as ATP-gated Ca2+ channels that facilitate Ca2+ influx, which is required for mitochondrial activity (28, 30). Therefore, we studied the role of P2X4 receptors in SDF-1α-induced Ca2+ signaling. The P2X4 receptor antagonist 5-BDBD reduced cytosolic Ca2+ signaling and completely abolished mitochondrial Ca2+ uptake and cellular ATP release in response to SDF-1α (Figure 4, A–E; and Supplemental Video 5). These findings suggest that endogenous P2X4 receptor stimulation promotes a feed-forward signaling mechanism that upregulates mitochondrial ATP production in response to SDF-1α. This is supported by the finding that blocking ATP release with CBX and inhibition of P2X4 receptors had similar effects on SDF-1α-induced Ca2+ signaling. CXCR4 stimulation is known to activate phosphatidylinositol 3-kinase (PI3K) signaling, which promotes the release of Ca2+ from intracellular stores (36). Treatment with the selective PI3K inhibitor wortmannin prevented the increase in intracellular Ca2+ following SDF-1α stimulation, which suggests that P2X4 receptors enhance and sustain mitochondrial activity following SDF-1α exposure (Figure 4, A–D).

**P2X4 receptors are needed for T cell polarization and pseudopod formation.** Leukocyte migration depends on the polarization of cells, which involves excitatory signals that promote actin polymerization and pseudopod protrusion at the leading edge (37). We hypothesized that P2X4 receptors provide such signals to enhance and amplify chemokine signaling and establish cell polarity in migrating T cells. We found that silencing of P2X4 receptors disrupts cell polarization and the migration of T cells in response to
SDF-1α (Figure 5, A–C; and Supplemental Video 6). In the absence of P2X4 receptor signaling, T cells assumed rounded shapes with a reduced cell surface area (Figure 5, A and D) and fewer pseudopodia (Figure 5E). Upon TCR stimulation, T cells transform into spontaneously migrating lymphoblasts that require P2X4 receptors for cell migration as shown by the fact that 5-BDBD dose-dependently and reversibly blocked the polarization and migration of TCR/CD28-stimulated T cells (Supplemental Figure 4; Figure 5; and Supplemental Video 6).

**P2X4 receptors regulate mitochondrial ATP production in migrating T cells.** In order to study the role of P2X4 receptors in cell migration, we examined the subcellular distribution of P2X4...
The Journal of Clinical Investigation

RESEARCH ARTICLE

Like others before, we also observed that active mitochondria frequently accumulate near the uropod of migrating cells where they are thought to fuel ATP-consuming, actin-myosin–driven cell contraction (Figure 6B) (38). We found that cell migration speed correlated with the proportion of mitochondrial mass near the uropod (Figure 6C and Supplemental Figure 5A). Slow-moving cells, however, used a portion of their mitochondria at the front to probe the extracellular Eaton–Lambert receptors using fluorescence-tagged P2X4 receptor fusion proteins expressed in Jurkat CD4+ T cells. In unstimulated cells, P2X4 receptors were expressed in puncta that were distributed throughout the plasma membrane. Cell stimulation and polarization resulted in a reorganized pattern with these P2X4 receptor–containing structures located primarily at the front of polarized cells. P2X4 receptors accumulated at sites of pseudopod protrusion that also featured increased mitochondrial activity (Figure 6A and Supplemental Video 7). Like others before, we also observed that active mitochondria frequently accumulate near the uropod of migrating cells where they are thought to fuel ATP-consuming, actin-myosin–driven cell contraction (Figure 6B) (38). We found that cell migration speed correlated with the proportion of mitochondrial mass near the uropod (Figure 6C and Supplemental Figure 5A). Slow-moving cells, however, used a portion of their mitochondria at the front to probe the extracel-
The subcellular localization of P2X4 receptors and the mitochondria that fuel these receptors has a central role in the regulation of T cell polarization and migration. This conclusion is further supported by our findings that the mitochondrial inhibitors CCCP, rotenone, and oligomycin impaired cell polarization, pseudopod formation, and cell migration (Supplemental Figure 5, B–E).

P2X4 receptor inhibition prevents T cell infiltration and lung allograft rejection. Cell migration is needed for the recruitment of alloreactive T cells into lung allograft tissues, where these cells cause acute and chronic transplant rejection (22). We tested whether pharmacological inhibition of P2X4 receptors prevents the infiltration of T cells into lung allografts using a mouse lung transplant model with major histocompatibility complex mismatched BALB/c (H-2d) donor lungs and C57BL/6 (H-2b) recipient mice. We chose this widely used model because it recapitulates the clinical situation where human leukocyte antigen (HLA) mismatches are unavoidable due to logistic constraints (39, 40). We
determined T cell recruitment into the lung allograft 24 hours after lung transplantation (i.e., at a time early enough to exclude the possibility that T cell proliferation interfered with cell numbers in allograft tissues). Treatment with the P2X4 receptor antagonist 5-BDBD reduced the accumulation of T cells in lung allografts, improved lung function as reflected in reduced peak airway pressures, and reduced the gross appearance of rejection when compared with allografts implanted into vehicle-treated control mice (Figure 7, A and B; and Supplemental Figure 6). Moreover, P2X4 receptor inhibition also blocked the proliferation of CD4+ and CD8+ C57BL/6 recipient T cells in mixed lymphocyte reactions with BALB/c donor splenocytes (Figure 7, C and D; and Supplemental Figure 6). The effect of suramin on mouse cells was less effective than its effect on human cells (Figure 3F). This may be due to species differences that have been previously reported for this inhibitor (41). Taken together, these data demonstrate that blocking P2X4 receptors can indeed prevent the recruitment and activation of alloreactive T cells in lung transplantation.

**Discussion**
T cell migration is an important but only partially understood lymphocyte function (2, 4, 5). In our previous work, we have shown that autocrine purinergic signaling mechanisms fine-tune the directed migration of neutrophils in a chemotactic gradient field (42–44). Chemotaxis is essential for neutrophils to pursue and eliminate invading microbes. T cells require a more complex repertoire of motility patterns to fulfill their diverse roles in host immune defense (3, 45). Several mathematical models have been developed to describe different migration modes such as Brownian motion, random walk, and Levy walk (4, 46, 47). However, the underlying molecular mechanisms that orchestrate these different migration patterns are incompletely understood.
SDF-1α is one of the chemokines involved in the migration of T cells in lung allotransplants and lymph nodes (11, 15, 48). We found that stimulation of the corresponding chemokine receptor, CXCR4, triggers rapid ATP release from CD4+ T cells and that the released ATP regulates T cell migration through autocrine stimulation of P2X4 receptors. Recently, we have shown that naive CD4+ T cells rely on basal autocrine purinergic signaling to maintain cell metabolism in a “stand-by” mode that is needed for immune surveillance (26). Release of low amounts of ATP from resting cells is sufficient to stimulate high-affinity P2X1 receptors that promote Ca²⁺ influx and maintain basal mitochondrial activity at levels needed for immune surveillance. Here we show that SDF-1α increases ATP release to the levels needed to stimulate P2X4 receptors that promote the transition of cells from the resting state to a “search mode.” Our results suggest that the localization of mitochondria is a key determinant of cell polarization,

Figure 5. P2X4 receptors regulate T cell polarization, pseudopod formation, and migration. (A–C) Cell migration after silencing of P2X4 receptors in Jurkat cells or pharmacological P2X4 inhibition (5-BDBD, 10 μM) in CD4+ T lymphoblasts in the presence or absence of SDF-1α. (A) Representative images of 4 experiments; ×20 objective; scale bar, 10 μm (see also Supplemental Video 6). (B) Jurkat cells were treated with control or P2X4-targeting siRNA at the indicated concentrations, and migration speed and range (in 30 minutes) were analyzed after 48 hours. Data represent mean ± SEM of 60 cells derived from 3 experiments. (C) Jurkat cells were transfected with control or P2X4-targeting siRNA (10 nM) and migration was analyzed after 48 hours. Data represent mean ± SD of 3 (Jurkat cells) or 4 (T cells) separate experiments each comprising 40 cells. (D) Effect of P2X4 silencing or inhibition on the cell surface area of Jurkat cells, primary CD4+ T cells, and CD4+ T lymphoblasts stimulated or not with SDF-1α. Box plots show the median and the distribution of 262, 553, and 276 Jurkat cells, 297, 290, and 127 primary CD4+ T cells, 290 control lymphoblasts, and 127 lymphoblasts treated with 5-BDBD. Cells were analyzed in 4 separate experiments. (E) Migration of primary CD4+ T cells (no stimulation) or CD4+ T lymphoblasts treated or not with 5-BDBD was monitored by time-lapse microscopy. The number of pseudopodia formed by a particular cell during the 30-minute observation period was recorded. Box plots show the median and the distribution of 212 (no stimulation), 140 (control), and 82 (P2X4 inhibitor) analyzed cells derived from 5 (no stimulation) or 3 independent experiments. *P < 0.05 vs. control (Kruskal-Wallis test). **P < 0.05 (1-way ANOVA); TCR, T cell receptor.
pseudopod formation, and cell migration. Mitochondrial accumulation near the uropod of migrating leukocytes has been reported before and may provide the ATP needed for cell migration (38). We found that translocation of a portion of the mitochondrial mass to the front allows cells to probe their environment in order to interact with other cells. Our findings suggest that mitochondria at the front are needed to fuel autocrine purinergic signaling through P2X4 receptors. Further studies will be needed to deter-

**Figure 6.** P2X4 receptors promote mitochondrial activation and localized ATP release from migrating T cells. (A) Distribution of EGFP-tagged P2X4 receptors and mitochondria in unstimulated and SDF-1α–stimulated Jurkat cells. Histograms show the distribution of P2X4 receptor fluorescence along the cell axis as indicated (rectangle) and represent mean ± SD of 7 independent experiments. Arrow indicates direction of migration (see also Supplemental Video 7); ×100 objective. Scale bar, 10 μm. (B) Mitochondria are in the back of fast-moving cells and translocate to the front of cells probing their surroundings or engaging with other cells. CD4+ T lymphoblasts stained with MitoTracker Red CM-H2Xros (top row; ×63 objective) or with MitoTracker and 2-2Zn (bottom row; ×100 objective) are shown. Images are representative of 30 (top) or 15 (bottom row) experiments. Arrows in cells probing their environment indicate spots of increased mitochondrial activity (see also Supplemental Video 8). Scale bar, 10 μm. (C) Migration speed and mitochondrial localization were analyzed in 30-second increments in cells derived from 5 different experiments. The results shown comprise 73 single experiments. (D) Localization of mitochondria in the front half of fast-moving, probing, or interacting cells. Data represent mean ± SD of 30 cells, derived from 7 separate experiments; *P < 0.05 (Kruskal-Wallis test). (E) Representative images (left) and fluorescence intensity traces (right) of mitochondrial activity (MitoTracker Red CM-H2Xros) in CD4+ T lymphoblasts before and after P2X4 receptor inhibition (5-BBD, 10 μM). Color coding was applied to demonstrate differences in mitochondrial activity. Right panel: Change in mitochondrial activity over time following treatment with 5-BBD or culture medium (control). Data are representative of 20 cells; ×100 objective (see also Supplemental Video 9). Scale bar, 10 μm. (F) Averaged mitochondrial activity (mean ± SEM) of 22 (control), 20 (P2X4 inhibitor), or 11 (apyrase; 10 U/ml) cells analyzed in 2 (apyrase) or 3 individual experiments; *P < 0.05 (1-way ANOVA).
mation lead to the clustering of active mitochondria with PANX1 channels and P2X1 and P2X4 receptors, which elicits a final boost of localized ATP release that leads to full-fledged T cell effector function (19, 20, 27).

Targeting T cell migration can prevent the influx of T cells into inflamed tissues and T cell activation in transplanted organs, which leads to pathological inflammatory responses, including allograft rejection. The potential of targeting chemokine receptors to attenuate allograft rejection has been explored before but targeting of a single chemokine has been shown to yield only marginal improvement (51). Our findings suggest that targeting P2X4 receptors that regulate T cell migration is a more effective strategy to attenuate T cell infiltration into allograft tissues.

Inhibition of P2X4 receptor signaling can also block T cell proliferation. Therefore, additional experiments will be necessary to determine the mechanisms that regulate the distribution of mitochondria in polarized and migrating T cells and to distinguish these mechanisms from other effects such as passive displacement by the nucleus.

Based on our findings, we propose the following mechanisms by which excitatory P2X4 receptors regulate T cell migration (Figure 7E). Stimulation of chemokine receptors (e.g., with SDF-1α) triggers downstream signaling that activates mitochondrial ATP production and ATP release through PANX1 channels. P2X4 receptors colocalize with mitochondria in clusters at the front of cells and facilitate Ca2+ influx to maintain local mitochondrial ATP production at the levels needed for cell migration. P2X4 receptor-induced Ca2+ signaling may also promote cytoskeletal remodeling that is required for cell migration, TCR signaling, and immune synapse formation (49, 50). Cell migration and immune synapse formation lead to the clustering of active mitochondria with PANX1 channels and P2X1 and P2X4 receptors, which elicits a final boost of localized ATP release that leads to full-fledged T cell effector function (19, 20, 27).

Targeting T cell migration can prevent the influx of T cells into inflamed tissues and T cell activation in transplanted organs, which leads to pathological inflammatory responses, including allograft rejection. The potential of targeting chemokine receptors to attenuate allograft rejection has been explored before but targeting of a single chemokine has been shown to yield only marginal improvement (51). Our findings suggest that targeting P2X4 receptors that regulate T cell migration is a more effective strategy to attenuate T cell infiltration into allograft tissues. Inhibition of P2X4 receptor signaling can also block T cell proliferation. Therefore, additional experiments will be necessary.
to determine potential side effects of long-term treatment with P2X4 receptor antagonists.

P2X4 receptors and related purinergic mechanisms involved in T cell migration may be promising therapeutic targets in lung transplantation, as suggested by previous work showing that P2X receptor antagonists could dramatically improve long-term graft survival in a mouse lung transplant model (52). Future studies will be needed to evaluate the pharmacokinetics of 5-BDBD, to refine currently used drug regimens (53, 54), and to study the efficacy of this and future P2X4 receptor antagonists in the prevention of chronic lung allograft rejection.

In summary, we conclude that P2X4 receptors are potential targets to modulate inflammatory processes in organ transplantation and in other inflammatory settings, such as in ischemia and reperfusion injury or inflammatory bowel disease.

Methods

Reagents. Fluo-4 AM, Rhod-2 AM, MitoTracker Red CM-H2Xros, CFSE, and CellTrace Far Red were purchased from Molecular Probes (Thermo Fisher Scientific). All antibodies used for flow cytometry experiments were primarily fluorochrome conjugated and purchased from Biologend. In particular, the following antibodies were used in this study: FITC anti-human CD69 (FN 50); APC anti-human CD4 (OKT4); FITC anti-mouse CD3 (17A2); PE anti-mouse CD4 (RM4-5); PerCP anti-mouse CD8 (53-6-7); PE anti-mouse CD8α (53-6-7); APC anti-mouse H-2Kb (AF6-88.5); anti-mouse CD16/CD32 (2.4G2, BD Pharmingen); anti-human CD3 (HIT3a, BD Pharmingen); anti-human CD8 (CD28.2, BD Pharmingen). SDF-1α was from R&D Systems. Suramin, NF023, NF279, 5-BDBD, and 10panx1 were from Thermo Fisher Scientific. All antibodies used for flow cytometry experiments were generated as previously described (20). Jurkat cells were transfected with 10 μg of the EGFP-P2X4 plasmid by electroporation with a Neon Transfection system (Thermo Fisher Scientific) according to the manufacturer’s instructions and cultured in RPMI medium supplemented with 10% heat-inactivated fetal calf serum for 5 hours. Cells were imaged with the Leica DMi6000B microscope mentioned above. Prior to imaging, EGFP-P2X4 receptor-expressing Jurkat cells were costained with MitoTracker Red CM-H2Xros (100 nM for 10 minutes) and stained for 5 minutes with 500 nM of a cell surface-targeting fluorescent ATP probe (2-2Zn, gift from Itaru Hamachi, Kyoto University, Kyoto, Japan) (23). Cells were treated with inhibitors as indicated and stimulated with SDF-1α (100 ng/ml). Fluorescence live-cell imaging was performed with an inverted Leica DMi6000B microscope (Leica Microsystems) equipped with a temperature-controlled (37°C) stage incubator (Live Cell Instrument) and a Leica DFC365 FX camera. Fluorescence images were captured through x63 or x100 oil objectives (NA 1.4) using TRITC and FITC filter sets (Leica Microsystems) and LeicaLAS microscope imaging software. Image analysis was done with ImageJ. To assess mitochondrial localization within polarized T cells, bright field and fluorescence image pairs of migrating cells were acquired. The leading and the trailing edges of each cell were determined by analyzing consecutive image sequences. Bright field images were used to determine cell shapes. Fluorescence images were used to determine the distribution of mitochondria in the front and back halves of each cell.

Transfection and P2X4 receptor distribution. Enhanced green fluorescent protein–tagged (EGFP-tagged) P2X4 receptor constructs were generated as previously described (20). Jurkat cells were transfected with 10 μg of the EGFP-P2X4 plasmid by electroporation with a Neon Transfection system (Thermo Fisher Scientific) according to the manufacturer’s instructions and cultured in RPMI medium supplemented with 10% heat-inactivated fetal calf serum for 5 hours. Cells were imaged with the Leica DMi6000B microscope mentioned above. Prior to imaging, EGFP-P2X4 receptor-expressing Jurkat cells were costained with MitoTracker Red CM-H2Xros (100 nM for 10 minutes at 37°C) to estimate colocalization of P2X4 receptors with mitochondria.

Silencing of PANX1 and P2X4 receptors. Targeting siRNAs and P2X4 receptors were purchased from Ambion (Silencer Select Pre-Designed siRNA, Thermo Fisher Scientific). A nontargeting siRNA (Qiagen) was included as a negative control in all experiments. Jurkat cells were transfected with 10 nM (final concentration) of the respective siRNAs with a Neon Transfection system (Thermo Fisher Scientific) and cultured for 48 hours as we had found reliable gene knockdown using these conditions (20).

ATP measurements. Freshly isolated CD4+ T cells or Jurkat cells (5 × 10^6, suspended in 150 μl RPMI medium) were incubated for 10 minutes with CBX, 10panx1, CCCP, or 5-BDBD as indicated, stimulated with SDF-1α (100 ng/ml) for the indicated times, and placed on ice to stop reactions. The supernatants were collected by centrifugation at 0°C and ATP concentrations were determined using a luciferin/luciferase reaction. The supernatants were collected by centrifugation at 0°C and ATP concentrations were determined using a luciferin/luciferase reaction.

**Live-cell imaging of calcium, mitochondria, and ATP release.** CD4+ T cells were attached to fibronectin-coated, glass-bottom chamber slides and stained with the cytosolic Ca^2+ indicator Fluo-4 AM (4 μM, 20 minutes), the mitochondrial Ca^2+ indicator Rhod-2 AM (1 μM, 10 minutes), or with the mitochondrial membrane potential and ROS-sensitive dye MitoTracker Red CM-H2Xros (100 nM, 10 minutes) in cell culture medium buffered with 20 mM HEPES. To image ATP release at the cell surface, cells were suspended in Hank’s balanced salt solution and stained with 5 minutes with 500 nM of a cell surface-targeting fluorescent ATP probe (2-2Zn, gift from Itaru Hamachi, Kyoto University, Kyoto, Japan) (23). Cells were treated with inhibitors as indicated and stimulated with SDF-1α (100 ng/ml). Fluorescence live-cell imaging was performed with an inverted Leica DMi6000B microscope (Leica Microsystems) equipped with a temperature-controlled (37°C) stage incubator (Live Cell Instrument) and a Leica DFC365 FX camera. Fluorescence images were captured through x63 or x100 oil objectives (NA 1.4) using TRITC and FITC filter sets (Leica Microsystems) and LeicaLAS microscope imaging software. Image analysis was done with ImageJ. To assess mitochondrial localization within polarized T cells, bright field and fluorescence image pairs of migrating cells were acquired. The leading and the trailing edges of each cell were determined by analyzing consecutive image sequences. Bright field images were used to determine cell shapes. Fluorescence images were used to determine the distribution of mitochondria in the front and back halves of each cell.

**Transfection and P2X4 receptor distribution.** Enhanced green fluorescent protein–tagged (EGFP-tagged) P2X4 receptor constructs were generated as previously described (20). Jurkat cells were transfected with 10 μg of the EGFP-P2X4 plasmid by electroporation with a Neon Transfection system (Thermo Fisher Scientific) according to the manufacturer’s instructions and cultured in RPMI medium supplemented with 10% heat-inactivated fetal calf serum for 5 hours. Cells were imaged with the Leica DMi6000B microscope mentioned above. Prior to imaging, EGFP-P2X4 receptor-expressing Jurkat cells were costained with MitoTracker Red CM-H2Xros (100 nM for 10 minutes at 37°C) to estimate colocalization of P2X4 receptors with mitochondria.

**Silencing of PANX1 and P2X4 receptors.** Targeting siRNAs and P2X4 receptors were purchased from Ambion (Silencer Select Pre-Designed siRNA, Thermo Fisher Scientific). A nontargeting siRNA (Qiagen) was included as a negative control in all experiments. Jurkat cells were transfected with 10 nM (final concentration) of the respective siRNAs with a Neon Transfection system (Thermo Fisher Scientific) and cultured for 48 hours as we had found reliable gene knockdown using these conditions (20).

**ATP measurements.** Freshly isolated CD4+ T cells or Jurkat cells (5 × 10^6, suspended in 150 μl RPMI medium) were incubated for 10 minutes with CBX, 10panx1, CCCP, or 5-BDBD as indicated, stimulated with SDF-1α (100 ng/ml) for the indicated times, and placed on ice to stop reactions. The supernatants were collected by centrifugation at 0°C and ATP concentrations were determined using a luciferin/luciferase ATP bioluminescence kit (Thermo Fisher Scientific).
CD3 antibodies (0.25 μg/ml) for 3 hours. CD69 expression in CD4^+ T cells was measured by flow cytometry (FACScalibur, BD Biosciences). Inhibitors as indicated were added during the duration of the experiment. Samples stimulated with anti-CD3/anti-CD28 antibody-coated beads, nonstimulated samples, and samples that had been depleted from monocytes by plastic adherence served as positive and negative controls. For proliferation studies, PBMCs were stained with CFSE following the manufacturer’s instructions and stimulated for 72 hours with anti-CD3 antibodies (0.25 μg/ml) in the presence of inhibitors as indicated. CD4^+ T cells were identified by forward and side scatter properties and by staining with anti-CD4 antibodies.

Coupling of T cells and antibody-coated beads. Freshly isolated human CD4^+ T cells (1 × 10^5) suspended in 200 μl fully supplemented cell culture medium were treated or not (control) with SDF-1 (10–6 M) for 10 minutes, mixed with 1 × 10^5 anti-CD3/anti-CD28 antibody-coated microbeads, and incubated in 1.5 ml microcentrifuge tubes at 37°C under gentle agitation in a shaking water bath. Coupling of T cells to beads was determined after 15 minutes using flow cytometry. Beads were identified by their fluorescence and forward and side scatter properties (Supplemental Figure 2C). Control samples were treated with latrunculin B (10 μM), an inhibitor of actin polymerization and immune synapse formation in T cells (56), to determine passive binding of cells and beads.

Mouse c57bl/6 and BALB/c mice were purchased from Jackson Laboratories and housed in accordance with institutional and NIH guidelines. For all experiments, male mice aged 8–10 weeks and weighing 20–25 g were used.

Mouse orthotopic lung transplantation. Orthotopic left vascularized lung transplants were performed as previously described using BALB/c (H-2b) or donor and c57bl/6 (H-2b) as recipient mice (46). Briefly, recipients were treated with the P2X4 inhibitor 5-BDBD (4.25 mg/kg, i.p.) or DMSO (vehicle control) 24 hours before and immediately after transplantation (53, 54, 57). Donor mice were treated with a single dose of 5-BDBD or DMSO 20 minutes prior to surgery. Donor mice were anesthetized with ketamine and xylazine (Patterson Veterinary), intubated, and ventilated with isoflurane/oxygen. Lungs were flushed with ice-cold PBS. Left lungs were harvested and cuffs were placed in the pulmonary artery, pulmonary vein, and bronchus. Left lung transplants into recipient mice were performed via thoracotomy and implantation of the cuffed hilar structures. Peak airway pressures of the transplanted lungs were measured to assess lung function 24 hours after transplantation as previously described (58). Transplanted lungs were harvested 24 hours after transplantation.

T cell infiltration into lung allografts. T cell recruitment into lung allografts was determined 24 hours after transplantation (i.e., at a time before T cell proliferation could affect cell numbers). Lung allografts were digested in 1 mg/ml collagenase D and 1 mg/ml DNase I (Roche, Sigma-Aldrich) for 1 hour at 37°C in a shaking water bath, filtered through a 70-μm cell strainer (Celltreat), and treated with RBC lysis buffer (Biolegend). Cells were treated with anti-mouse CD16/CD32 antibodies (mouse BD Fc block) for 10 minutes and stained with antibodies against CD3, CD4, CD8, and H-2K^d. The number of recipient CD4^+ and CD8^+ T cells (H-2K^d CD3^+) was determined by flow cytometry using Precision Count Beads (Biolegend).

In vitro mixed lymphocyte reactions. Splenocytes were isolated using standard procedures. In vitro mixed lymphocyte reactions were performed in round-bottom 96-well plates using 4 × 10^5 CellTrace Far Red-labeled c57bl/6 (responder) splenocytes and 6 × 10^4 CFSE-labeled, irradiated (10 Gy) BALB/c (stimulator) splenocytes. CCCP and P2 receptor inhibitors were added to the cocultures for the duration of the experiment as indicated. Proliferation of CD4^+ or CD8^+ responder T cells was evaluated by flow cytometry after 4 days.

Statistics. Data are mean ± SD unless otherwise stated. Data were tested for normality with the Shapiro-Wilk test. Differences among normally distributed groups were tested for statistical significance using a 2-tailed unpaired Student’s t test or 1-way ANOVA followed by post hoc Holm-Sidak test if 2 or multiple groups were compared, respectively. Nonparametric Mann-Whitney U test or Kruskal-Wallis test followed by post hoc Dunn’s test were used for 2 or multiple group comparisons, respectively, when the normality test failed. Pearson’s correlation analysis was used to test if parameters were correlated. Differences were considered statistically significant at P < 0.05.

Study approval. All studies involving human subjects were approved by the IRB of Beth Israel Deaconess Medical Center and written informed consent was obtained before blood draw. All procedures involving animals were approved by the IACUC of Beth Israel Deaconess Medical Center and Boston Children’s Hospital.

Author contributions. WJG developed the overall study design and supervised the project. CL designed, performed, and analyzed experiments. KL performed lung transplant surgery. TD, MA, and MF assisted with flow cytometry experiments. YK and CJS provided critical technical assistance. SD, JH, and KK helped with the analysis of T cell motility data. JAL, SCR, and GAV provided helpful discussions, provided experimental support, and assisted with the preparation of the manuscript. GAV helped with the design of the lung transplant experiments. CL and WJG prepared the manuscript with input from the other authors.

Acknowledgments. This work was funded in part by grants GM-51477, GM-60475, GM-116162, AI-080582, and T32 GM-103702 from the National Institutes of Health (to WJG).

Address correspondence to: Wolfgang G. Junger, Harvard Medical School, Beth Israel Deaconess Medical Center, Department of Surgery, 330 Brookline Avenue, Boston, Massachusetts 02215, USA. Phone: 617.667.7415; Email: wjunger@bidmc.harvard.edu.


jci.org