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P2Y11 receptors support T cell activation by directing mitochondrial trafficking to the immune synapse

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Abstract

T cells form an immune synapse (IS) with antigen-presenting cells (APCs) to detect antigens that match their T cell receptor (TCR). Mitochondria, pannexin-1 (panx1) channels, and P2X4 receptors congregate at the IS where mitochondria produce the ATP that panx1 channels release in order to stimulate P2X4 receptors. P2X4 receptor stimulation causes cellular Ca^{2+} influx that upregulates mitochondrial metabolism and localized ATP production at the IS. Here we show that P2Y11 receptors are essential players that sustain these T cell activation mechanisms. We found that P2Y11 receptors retract from the IS towards the back of cells where their stimulation by extracellular ATP induces cAMP/PKA signaling that redirects mitochondrial trafficking to the IS. P2Y11 receptors thus reinforce IS signaling by promoting the aggregation of mitochondria with panx1 ATP release channels and P2X4 receptors at the IS. This dual purinergic signaling mechanism involving P2X4 and P2Y11 receptors focuses mitochondrial metabolism to the IS where localized ATP production sustains synaptic activity in order to allow successful completion of T cell activation responses. Our findings have practical implications because rodents lack P2Y11 receptors, raising concerns as to the validity of rodent models to study treatment of infections and inflammatory conditions.

Graphical Abstract

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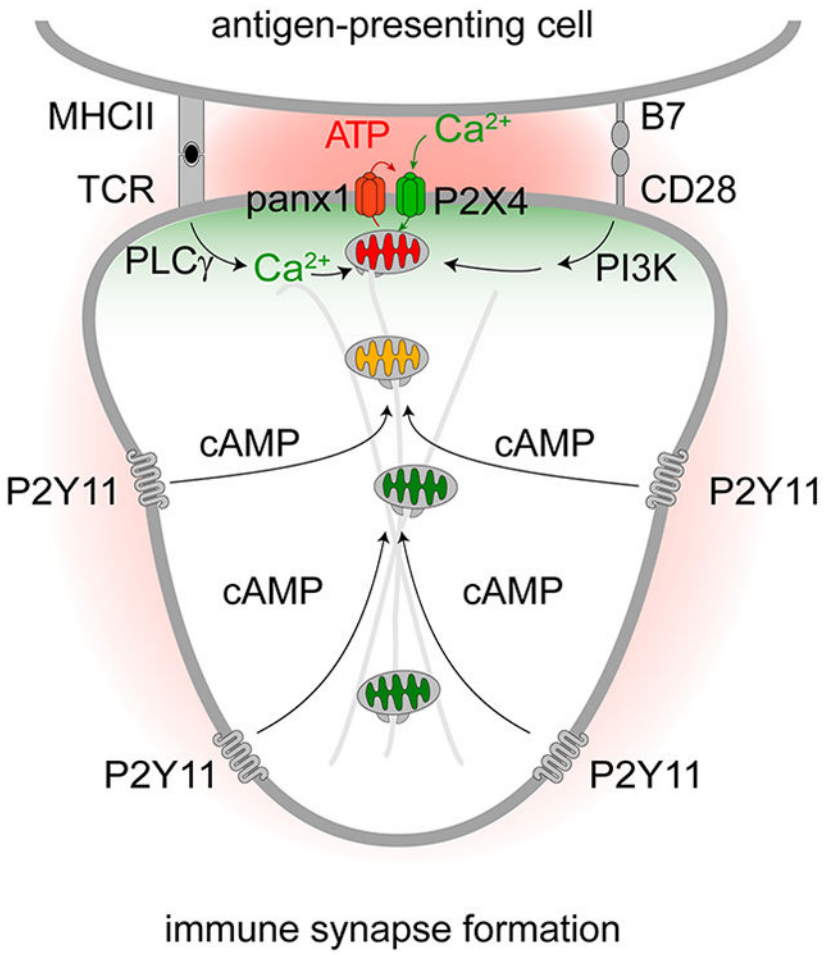
AUTHORSHIP

W.G.J. conceptualized the study. C.L., S.B., C.J.S, and K.S. performed and analyzed experiments. C.L. and W.G.J. wrote the paper.

DISCLOSURES

The authors declare no conflict of interests.



Summary sentence:

Autocrine stimulation of P2Y11 receptors induces the transport of mitochondria to the immune synapse where they generate the ATP needed to fuel T cell activation.

Keywords

CD4 T cells; mitochondrial translocation; immune synapse; purinergic signaling

INTRODUCTION

ATP is the main energy carrier that fuels most cellular processes within living cells. Under specific circumstances, however, cells can also release a portion of that ATP into the extracellular space. ATP release can occur through different types of membrane channels, via exocytotic pathways, or as a result of cell damage that ruptures cell membranes.[1-3] Extracellular ATP can act as a signaling molecule that binds to specific cell surface receptors termed purinergic receptors.[4] These purinergic receptors are found on virtually all mammalian cells. The human purinergic receptor family comprises seven P2X receptor subtypes that are ATP-gated cation channels and eight P2Y receptors that belong to

the G-protein coupled receptor (GPCR) superfamily and recognize ATP, ADP, and other nucleotides. Released ATP can also be hydrolyzed to adenosine that activates the P1 adenosine receptor family members. P1 receptors consist of four different subtypes, namely the A1, A2a, A2b, and A3 receptors that all belong to the GPCR superfamily.[5-7]

Geoffrey Burnstock was first to describe the concept of purinergic signaling.[8] He and his team found that ATP released from nerve cells acts as a co-transmitter at neuronal synapses. Similar purinergic signaling mechanisms were subsequently identified in many other organ systems and shown to regulate a wide range of mammalian cell types including the various different immune cell subpopulations.[9-12] CD4 positive T cells (CD4 cells) are important components of the cellular immune system that coordinate adaptive immune responses.[13] Purinergic signaling regulates key aspects of CD4 cell biology, including cell migration, T cell receptor (TCR) signaling, and the proliferation and differentiation of these T cells.[14-20] The stimulation of CD4 cells requires their cellular interaction with antigen-presenting cells (APCs) whereby CD4 cells use their TCR to probe the antigens displayed on the surface of APCs. These intercellular interactions are dynamic in nature and permit CD4 cells to assess and compare the affinities of their TCR for different antigens presented by APCs in lymphoid organs. A successful match with an antigen exhibited by an APC leads to the formation of a specialized signaling structure referred to as an immune synapse (IS). The IS of T cells comprises microclusters of TCR, CD3, CD28 coreceptors, LFA-1, LAT, SLP76, and other molecules that interact with corresponding APC binding partners like pMHCs, CD80/CD86, and ICAM1.[21,22]

Similar to neuronal synapses, the IS also involves the translocation of mitochondria to the contact site that T cells form with APCs. The mitochondria at the IS regulate cytosolic Ca^{2+} levels and produce cellular ATP that T cells release into the synaptic cleft.[23-25] Pannexin-1 (panx1) channels and P2X4 receptors also accumulate at the IS, where panx1 channels facilitate the release of ATP that stimulates P2X4 receptors. P2X4 receptors, in turn, mediate Ca^{2+} influx from the extracellular space, which upregulates mitochondrial metabolism at the IS.[16,18] Localized ATP release, P2X4 receptor signaling, and Ca^{2+} influx at the IS collectively fuel mitochondrial ATP production and supply T cells with the energy needed to execute subsequent steps involved in T cell activation.[16,25]

P2X4 receptors have a central role in these processes because they regulate T cell migration and trigger cellular Ca^{2+} influx that are prerequisites for the T cell/APC encounters that initiate antigen-induced T cell responses.[15] In addition to P2X4 receptors, human T cells also express P2Y11 receptors.[26] However, the role of P2Y11 receptors in T cell activation is still not clearly understood. Like P2X4 receptors, P2Y11 receptors are designated ATP receptors. Instead of facilitating Ca^{2+} influx, though, P2Y11 receptors are GPCRs known to couple to $G\alpha_s$ proteins that trigger cAMP/PKA signaling and modulate T cell functions.[27,28] Here we investigated whether and how P2Y11 receptors contribute to the interaction of T cells with APCs and the subsequent downstream signaling events that culminate in T cell activation.

MATERIALS AND METHODS

Reagents

Carboxyfluorescein succinimidyl ester (CFSE), Fluo-4 AM, MitoTracker Red CM-H2Xros, MitoTracker Green FM, and Tubulin Tracker Deep Red were purchased from Thermo Fisher Scientific (Waltham, MA). The P2Y11 receptor antagonist NF340, the P2Y11 receptor agonist NF546, the P2X4 receptor antagonist 5-BDBD, the PKA inhibitor H89, and nocodazole were from R&D Systems (Minneapolis, MN), while cell permeable cAMP-AM was from BIOLOG Life Science Institute (Bremen, Germany). Anti-human CD4-allophycocyanin (clone: OKT4), anti-human CD69 FITC (clone: FN50), and anti-human CD11b Alexa Fluor 488 (clone: ICRF44) antibodies were purchased from Biolegend (San Diego, CA). Mouse anti-human CD3 (clone: HIT3a) and anti-human CD28 (clone; CD28.2) antibodies were from BD Pharmingen (Franklin Lakes, NJ) and goat anti-mouse IgG Fc antibodies were from Pierce (Thermo Fisher Scientific). Latrunculin B and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Cell isolation and cell culture

The Institutional Review Board of the Beth Israel Deaconess Medical Center approved all studies involving human subjects and written informed consent was obtained prior to blood draws. PBMCs were isolated from the heparinized whole blood of healthy volunteers by Ficoll-Paque (GE Healthcare, Chicago, IL) density centrifugation. CD4 cells were isolated from PBMCs by positive selection using immunomagnetic beads (Miltenyi Biotec, Auburn, CA). Cells were maintained in RPMI-1640 medium (HyClone, Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Thermo Fisher Scientific).

Jurkat T cells (clone E6-1) and U-937 cells were cultured in fully supplemented RPMI-1640 medium as previously described.[25] Phoenix-Ampho cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA) at 37°C in 5% CO_2. The cell lines were purchased from the American Type Culture Collection (Manassas, VA) that certified their authenticities and absence of contaminants. All experiments were performed with cells from low (<13) passage cultures.

Transfection and retroviral infection

The P2X4 receptor construct tagged with enhanced green fluorescent protein (EGFP) was generated as previously described.[18] The pBMN-P2Y11-YFP plasmid was a kind gift from Severin Mühleder and Wolfgang Holnthoner (Ludwig Boltzmann Institute for Clinical and Experimental Traumatology, Vienna, Austria).[29] The CMV-mito-CAR-Geco plasmid was obtained from Addgene (#46022; Addgene, Cambridge, MA).[30] A mixture of four siRNAs targeting the P2Y11 receptor (SMARTpool® siRNA) was purchased from Dharmacon (Lafayette, CO). Jurkat cells were transfected with the EGFP-P2X4 plasmid (10 μg), the CMV-mito-CAR-Geco plasmid (10 μg), P2Y11 targeting siRNA, or non-targeting control siRNA (20 nM final concentration) using a Neon transfection system (Invitrogen). Silencing

efficiency was confirmed by qPCR as previously described using predesigned primers for the reference gene TATA box binding protein (*TBP*; QuantiTect primer assay, Qiagen, Valencia, CA) and the following primers targeting the P2Y11 receptor: *P2YR11* sense: 5'-GTT GGT GGC CAG TGG TGT G-3'; *P2YR11* anti-sense: 5'-TTG AGC ACC CGC ATG ATG T-3' (Fig. S1A).[31] Cells were cultured 5 h (plasmids) or 24 h (siRNA) after transfection in culture medium without antibiotics. Phoenix-Ampho cells were transfected with 10 μg of the pBMN-P2Y11-YFP plasmid using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Virus supernatants were collected after 48 and 72 h. Jurkat cells were resuspended in fresh virus supernatants containing 2 μg/ml polybrene (Santa Cruz Biotechnology, Santa Cruz, CA), centrifuged at 400 x *g* and room temperature for 1.5 h, and incubated in a cell culture incubator overnight. After 24 h, cells were infected a second time and cultured for another 48 to 72 h before imaging experiments were performed.

T cell proliferation

PBMCs were labeled with CFSE (ThermoFisher Scientific), pretreated with 5-BDBD (10 μM), NF340 (10 μM), H89 (5 μM), or vehicle control (0.1% DMSO in cell culture medium) for 10 min and stimulated for 72 h with soluble anti-CD3 antibodies (0.25 μg/ml). These stimulation conditions ensured that T cells interact with accessory cells in order to obtain the necessary costimulatory signals needed for complete T cell activation. CD4 cells were identified based on their forward and side scatter properties and by anti-CD4 antibody labeling. The percentage of proliferating ($CFSE^{low}$) CD4 cells was determined using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

CD69 expression and IL-2 production

PBMCs ($1.5x10^5$ per well) were stimulated in 96-well cell culture plates with anti-CD3 antibodies (0.25 μg/ml) in the presence or absence of 5-BDBD (10 μM), NF340 (10 μM), H89 (5 μM), or vehicle control. After 4 h, CD69 expression of CD4 cell cultures was measured by flow cytometry. IL-2 concentrations in culture supernatants were determined 6 h after cell stimulation using a commercially available ELISA assay kit (R&D Systems).

T cell-monocyte conjugates

PBMCs were tagged with allophycocyanin-labeled anti-CD4 and Alexa Fluor 488-labeled anti-CD11b antibodies and preincubated for 10 min with latrunculin B (10 μM), 5-BDBD (10 μM), NF340 (10 μM), H89 (5 μM), or vehicle control. Then, anti-CD3 antibody solution (0.25 μg/ml) or vehicle control (unstimulated controls) was added and cells were briefly pelleted at 100 x *g* in order to facilitate conjugate formation between T cells and monocytes. After a 1-h incubation period at 37°C, the percentages of CD4+ T cells that had formed conjugates with CD11b+ monocytes were analyzed with flow cytometry.

ATP measurements

Jurkat cells ($5x10^5$, suspended in 150 μl culture medium in microcentrifuge tubes) were incubated for 30 min at 37°C with 5-BDBD (10 μM), NF340 (10 μM), or vehicle control. Anti-CD3/CD28 antibody-coated microbeads (1 bead per cell; Dynabeads, Thermo Fisher

Scientific) were added and reactions stopped after 0.5 or 5 min by freezing cells in liquid nitrogen. Cells were thawed, mixed with perchloric acid (0.4 M) to prevent enzymatic breakdown of ATP, and homogenized by sonication. ATP concentrations in pH-neutralized cell extracts were determined using a luciferin/luciferase-based ATP bioluminescence assay kit (Thermo Fisher Scientific).

Mitochondrial translocation

Jurkat T cells were labeled with MitoTracker Red CM-H2Xros and monocyte-like U-937 cells were labeled with MitoTracker Green FM. Jurkat and U-937 cells were mixed at a ratio of 1:1 and allowed to attach to fibronectin-coated glass-bottom chamber slides (Nunc Lab Tek, Thermo Fisher Scientific). Cells were reconstituted in phenol red-free cell culture medium that was buffered with 20 mM HEPES and treated for 20 min with nocodazole (5 μM), 5-BDBD (10 μM), NF340 (10 μM), H89 (5 μM), or vehicle control. Cells were then stimulated with anti-CD3 antibodies (0.25 μg/ml) for another 15 min in a temperature-controlled (37°C) stage incubator (Live Cell Instrument, Seoul, South Korea). Conjugate formation and distribution of mitochondria within Jurkat cells was analyzed by fluorescence microscopy using a Leica DMI6000B inverted microscope equipped with a DFC365 FX camera and Leica LAS X imaging software (Leica Microsystems, Wetzlar, Germany). TRITC and FITC excitation/emission filters sets were used to identify mitochondria in Jurkat cells and U-937 cells, respectively. Bright field images were simultaneously acquired to record cell outlines, to quantify the percentages of Jurkat cells that formed conjugates with U-937 cells, and to assess accumulation of Jurkat cell mitochondria at the cell-to-cell contact sites between T cells and APCs. Cell shapes and mitochondrial distribution patterns were compared and evaluated with NIH ImageJ software. Mitochondria were judged to have accumulated at the IS when >70% of the total mitochondrial mass was located in the front half section of a T cell.

Live-cell imaging of purinergic receptors, mitochondria, microtubules, and assessment of mitochondrial trafficking

The expression patterns of P2X4 and P2Y11 receptors were analyzed in Jurkat cells expressing EGFP-P2X4 or YFP-P2Y11 receptor fusion proteins. Cells were stimulated with anti-CD3/CD28 antibody-coated microbeads and the distribution patterns of P2Y11 and P2X4 receptors were assessed with fluorescence microscopy and ImageJ software before and up to 15 min after stimulation of cells with microbeads. When indicated, cells were treated with the P2Y11 receptor antagonist NF340 (10 μM) for 10 min prior to cell stimulation. To study the association of mitochondria with microtubules, Jurkat cells were labeled for 30 min with MitoTracker Red CM-H2Xros (100 nM) and Tubulin Tracker Deep Red (1 μM) at 37°C. Then, bright field and fluorescence image pairs were captured through a 100x oil objective (Leica, NA 1.4) using TRITC and Cy5 filter sets. Mitochondrial movements were analyzed using Jurkat cells that expressed the mitochondrial indicator mito-CAR-Geco. These cells were allowed to attach to fibronectin-coated cover glass dishes, washed with pH-buffered phenol red-free cell culture medium, and treated with agonists or antagonists as indicated. In some experiments, cells were stimulated with anti-CD3/CD28 antibody-coated microbeads. Mitochondrial movement was recorded for up to 4.5 min using time-lapse fluorescence microscopy and TRITC excitation/emission filters as well as a 100x

oil objective (Leica, NA 1.4). Migration paths of single mitochondria or distinct segments of the mitochondrial network that were clearly identifiable in each image were tracked with an ImageJ MTrackJ plugin in order to calculate the speed of mitochondrial movement. Traces of individual mitochondrial paths were compared by aligning the origins of these paths at coordinate x=y=0. The paths of multiple mitochondria were displayed in spider plots. In these plots, the ultimate destinations, i.e., IS-inducing microbeads were assigned the coordinates x=0 and y=7. Mitochondria were categorized as showing directional movement if the angle of their migration paths did not deviate by more than 30° from a straight line towards the IS located at coordinate x=0, y=7.

Calcium measurements

CD4 cells were labeled for 30 min with the cytosolic Ca^{2+} probe Fluo-4 AM (4 μM) at 37°C. Imaging was carried out with cells attached to fibronectin-coated glass bottom dishes. Cells were treated for 10 min with 5-BDBD (10 μM), NF340 (10 μM), or vehicle control and stimulated by TCR/CD28 crosslinking with mouse anti-human CD3, mouse anti-human CD28, and anti-mouse IgG Fc antibodies (0.25 μg/ml of each antibody). Anti-CD3 and anti-CD28 antibodies were added 30 s before anti-IgG antibodies. TCR/CD28 crosslinking rather than beads was used for cell stimulation to enable simultaneous acquisition of statistically meaningful events, even though this strategy did not ensure authentic immune synapse formation. Changes in fluorescence readouts were recorded at a rate of 20 frames/min using a FITC filter set and the Leica DMI6000B microscope described above (40x objective, NA 0.75). Mitochondrial Ca^{2+} signaling was recorded in Jurkat cells expressing the mitochondrial Ca^{2+} sensor mito-CAR-Geco. Jurkat cells were preincubated with NF340 (10 μM) or H89 (5 μM) as indicated and IS formation was induced by stimulation with anti-CD3/CD28 antibody-coated microbeads. Then cells were imaged at a rate of 12 frames/min using a TRITC filter set and a 100x oil objective (Leica, NA 1.4). Changes in fluorescence values of single cells (cytosolic Ca^{2+}) or single mitochondria (mitochondrial Ca^{2+}) were analyzed over time with ImageJ software.

Statistical analyses

Superimposed data points indicate results of individual experiments. Results of two groups were compared with unpaired two-tailed Student’s t tests. One-way ANOVA followed by Holm-Sidak post-hoc test was used for multiple group comparisons. Not normally distributed data were analyzed using the Mann-Whitney U test (two groups) or the Kruskal-Wallis test followed by Dunn’s post-hoc test (multiple group comparisons). Differences were considered statistically significant at $P<0.05$.

RESULTS

Autocrine signaling via P2Y11 receptors is involved in CD4 cell activation

We have previously shown that cellular ATP release and autocrine signaling through P2X4 receptors are important prerequisites for T cell activation.[15,18] The role of P2Y11 receptors in T cell activation is not well defined. Therefore, we compared the relative contributions of P2X4 and P2Y11 receptors to T cell activation. We stimulated human peripheral blood mononuclear cell (PBMC) cultures with anti-CD3 antibodies, which requires that CD4

cells interact with APCs in order to obtain the necessary costimulatory signal by CD28 coreceptor engagement that leads to full-fletched T cell responses. As previously shown, pretreatment with the P2X4 receptor antagonist 5-BDBD blocked the proliferation of CD4 cells under those conditions.[15] However, the P2Y11 receptor antagonist NF340 also reduced cell proliferation, which indicates that P2X4 and P2Y11 receptors are both required for CD4 cell activation (Fig. 1A-B). P2Y11 receptor inhibition not only impaired T cell proliferation but also suppressed CD69 expression and IL-2 production in response to T cell stimulation (Fig. 1C-D). These results indicate that autocrine stimulation of P2Y11 receptors is involved in the early T cell activation events needed for T cell proliferation. P2Y11 receptors are GPCRs known to couple to $G\alpha_s$ proteins that increase intracellular cAMP levels and trigger PKA signaling.[27] Blocking PKA signaling with H89 replicated the suppressive effect of P2Y11 receptor antagonism on T cell activation (Fig. 1A-D). Based on these findings, we concluded that autocrine stimulation of P2Y11 receptors and downstream cAMP/PKA signaling are important components of the T cell activation cascade.

P2X4 receptors trigger and P2Y11 receptors sustain the Ca^{2+} signal needed for T cell activation

IS formation is associated with intracellular Ca^{2+} signaling events that initiate T cell stimulation and subsequent processes that culminate in functional T cell responses.[32] These Ca^{2+} signaling events involve P2X4 receptors that induce Ca^{2+} influx in response to ATP stimulation.[15,18] However, it is not known whether and how P2Y11 receptors contribute to IS formation and Ca^{2+} signaling. In order to study whether P2Y11 receptors are involved in Ca^{2+} signaling during T cell stimulation, we loaded human CD4 cells with the cytosolic Ca^{2+} probe Fluo-4 AM that allows real-time monitoring of intracellular Ca^{2+} levels using live-cell fluorescence microscopy. In agreement with previous reports[18,33], we found that inhibition of P2X4 receptors completely abolished the Ca^{2+} signaling response to cell stimulation by TCR/CD28 crosslinking (Fig. 2A-B). Interestingly, however, inhibition of P2Y11 receptors also diminished Ca^{2+} signaling in response to TCR/CD28 stimulation. While P2X4 receptor blockade abolished the initiation and the subsequent sustained phases of Ca^{2+} signaling, P2Y11 receptor blockade attenuated primarily the sustained Ca^{2+} signaling phase (Fig. 2B-C). These findings indicate that P2X4 and P2Y11 receptors are both necessary for Ca^{2+} signaling but that these different purinergic receptors facilitate distinct aspects of the Ca^{2+} signaling mechanism that culminates in full-fletched T cell activation responses.

P2X4 and P2Y11 receptors sustain T cell metabolism following immune synapse formation

P2X4 receptors contribute to T cell activation by facilitating localized Ca^{2+} influx that drives the mitochondrial metabolism needed to produce the ATP that is released into the IS.[15,18,34] However, the contribution of P2Y11 receptors to this signaling process is unknown. In order to test whether P2Y11 receptors contribute to the interaction of CD4 cells with APCs, we stimulated PBMCs with anti-CD3 antibodies in the presence or absence of P2Y11 or P2X4 receptor antagonists or the PKA inhibitor H89. First, we studied whether these treatments influence the physical association of CD4 cells with APCs. TCR stimulation caused a significant increase in the percentage of CD4 cells that formed stable conjugates with APCs (Fig. 3A-B). Pretreatment with P2X4 or P2Y11 receptor antagonist

or with the PKA inhibitor had no significant influence on the formation of these CD4 cell/APC conjugates as determined by flow cytometry (Fig. 3B) or by direct assessment via fluorescence microscopy (Fig. S1B). However, disruption of actin polymerization with latrunculin B completely prevented the interaction of CD4 cells with APCs (Fig. 3B). These findings suggest that T cells require neither P2X4 nor P2Y11 receptors to interact and form cell-to-cell contacts with APCs. However, T cell activation requires more than mere cell-to-cell-contacts with APCs. Instead, T cells must form functional immune synapses that are characterized by localized ATP release and autocrine purinergic signaling in order to induce cellular Ca^{2+} influx and mitochondrial metabolism to fuel subsequent signaling processes required for T cell activation.[16,23,25] Therefore, we studied whether P2Y11 receptor signaling is required for the upregulation of cell metabolism in response to TCR/CD28 stimulation. For that purpose, we stimulated Jurkat CD4 T cells with microbeads coated with anti-CD3 and anti-CD28 antibodies in order to simulate cell stimulation by APCs. The intracellular ATP content of Jurkat cells rapidly increased in response to stimulation with these microbeads, reaching near plateau levels as early as 30 seconds after TCR/CD28 stimulation and further increasing over the next 5 min (Fig. 3C). Treatment with the P2X4 or P2Y11 receptor antagonists had little effect on the initial spike in ATP production but both antagonists caused ATP levels to drop back to resting levels within 5 min after T cell stimulation (Fig. 3C). Taken together, these findings indicate that neither P2X4 nor P2Y11 receptors are required for the initial burst of cell metabolism that occurs following the physical interaction of T cells with APCs. However, our findings suggest that both P2X4 and P2Y11 receptors are necessary to sustain the metabolic program that allows T cells to complete the cell activation process that must follow their formation of an IS with APCs.

P2Y11 receptors and microtubules direct mitochondria to the immune synapse

The findings above demonstrate that P2X4 and P2Y11 receptors are required to maintain cellular ATP production and sustain T cell activation following TCR/CD28 stimulation at the IS. An important prerequisite for sustained T cell activation is the accumulation of mitochondria at the IS.[23] Mitochondria regulate local Ca^{2+} homeostasis at the IS and produce the ATP needed to ignite and sustain signaling mechanisms that culminate in cell responses such as IL-2 production and T cell proliferation.[24,25,35] We have previously shown that P2X4 receptors contribute to mitochondrial metabolism at the IS.[18,31] We wondered whether P2Y11 receptors are also involved in the activation of mitochondrial metabolism at the IS. In order to address this question, we co-cultured Jurkat CD4 T cells with U-937 monocytes that served as APCs. To distinguish both cell types from one another, we labeled Jurkat cells with MitoTracker Red and U-937 cells with MitoTracker Green. This also allowed monitoring of mitochondrial trafficking during IS formation using live-cell fluorescence imaging. Stimulation with soluble anti-CD3 antibodies induced rapid formation of cell-to-cell conjugates between Jurkat cells and U-937 cells. Jurkat cells produced immune synapses featuring mitochondrial accumulation at the cell-to-cell contact sites with the APCs (Fig. 4A, Video 1). Inhibition of P2Y11 receptors or of PKA, or silencing of P2Y11 receptors with siRNA impaired mitochondrial trafficking to the IS (Fig. 4A-B, Video 1). Mitochondrial trafficking to the IS was also abolished by disrupting microtubule polymerization with nocodazole or by inhibition of P2X4 receptors (Fig. 4A-B). These data

demonstrate that autocrine signaling via P2X4 and P2Y11 receptors and the microtubule (MT) network are essential requirements for the translocation of mitochondria to the IS of T cells.

P2Y11 receptor-induced cAMP signaling promotes mitochondrial trafficking within T cells

In neurons and other mammalian cells, kinesin and dynein motors transport mitochondria along the MT network.[36,37] Similar mechanisms may regulate mitochondrial trafficking in T cells.[38,39] Therefore, we hypothesized that P2Y11 receptor signaling may be involved in mitochondrial trafficking along the MT network of activated T cells. In order to test this hypothesis, we expressed the mitochondrial Ca^{2+} sensor mito-CAR-Geco in Jurkat cells and labeled the MT network with Tubulin Tracker Deep Red. Live-cell video imaging of these cells revealed that mitochondria remain closely associated with the MT network in T cells. In addition, mitochondria apparently associated with the microtubule organizing center (MTOC) that is known to translocate to the IS of stimulated T cells (Fig. 5A).[40] In T cells stimulated with anti-CD3/CD28 antibody-coated microbeads, trafficking along the MT network resulted in rapid accumulation of mitochondria at the site of IS formation. Mitochondrial trafficking rapidly increased in cells treated with the P2Y11 receptor agonist NF546 and in cells loaded with permeable cAMP-AM. However, these treatments induced disorganized mitochondrial trafficking and mitochondria remained randomly distributed throughout the cytosol of these cells (Video 2; Fig. 5B). P2Y11 receptor stimulation triggered mitochondrial trafficking within seconds after the addition of the P2Y11 receptor agonist NF546 (Fig. 5C). This response was faster than the mitochondrial trafficking triggered by cell stimulation with anti-CD3/CD28 antibody-coated microbeads. However, both modes of cell stimulation resulted in similar peak mitochondrial motility speeds (Fig. 5C-D). While P2Y11 receptor agonist stimulation induced a random mitochondrial motility pattern, the mitochondrial network in bead-stimulated T cells showed a highly directional movement towards the site of IS formation (Video 2; Fig. 5B and E). Taken together, these findings indicate that P2Y11 receptor signaling increases mitochondrial trafficking in a cAMP and MT-dependent manner that leads to the accumulation of mitochondria at the IS.

P2Y11 receptors drive mitochondria from the back to the immune synapse at the front of cells

Panx1 channels and P2X4 receptors accumulate with mitochondria at the IS, resulting in Ca^{2+} influx that fuels mitochondrial metabolism and produces localized ATP release that stimulates P2X4 receptor feedback signaling at the IS.[16,18,25] The findings above indicated that P2Y11 receptors induce cAMP signaling that propels mitochondria along the MT network to the IS of stimulated T cells. We hypothesized that redistribution of P2Y11 receptors across the cell surface may help direct mitochondria to the IS of stimulated cells. In order to test this possibility, we compared the redistribution of P2X4 and P2Y11 receptors in response to IS formation of T cells. We expressed YFP-tagged P2Y11 or EGFP-tagged P2X4 receptor fusion proteins in Jurkat T cells and stimulated these cells with anti-CD3/CD28 antibody-coated microbeads. We examined the redistribution of the fluorescence-tagged receptors using fluorescence microscopy. As previously reported, we found that P2X4 receptors were trafficked to the cell surface and accumulated at the IS following T cell stimulation (Fig. 6A).[18] Inhibition of P2Y11 receptors did not prevent that

accumulation of P2X4 receptors at the IS, which indicates that P2X4 receptor trafficking is independent of P2Y11 receptor-mediated mitochondrial trafficking (Video 3, Fig. S2A-B). In contrast to P2X4 receptors, however, we found that P2Y11 receptors were uniformly distributed across the cell surface of unstimulated T cells and that they were displaced from membrane regions associated with IS formation following T cell stimulation (Fig. 6B).

These findings suggested that P2Y11 receptors redistribute from the IS towards the back of cells where they induce cAMP signaling that directs the movement of mitochondria to the IS. The resulting proximity of mitochondria and P2X4 receptors facilitates localized Ca^{2+} uptake that increases mitochondrial activity at the IS. In order to test this concept, we expressed the mitochondrial Ca^{2+} sensor mito-CAR-Geco in Jurkat cells and studied how P2Y11 receptor signaling affects the translocation and activation of mitochondria during IS formation. Cells that interacted with anti-CD3/CD28 antibody-coated microbeads rapidly responded by trafficking their mitochondria to the site of IS formation (Fig. 7A-C, Video 4). This process was paralleled by a rapid increase in mitochondrial Ca^{2+} influx, which is indicative of mitochondrial metabolism activation in response to IS formation (Fig. 7D, Video 4). In addition, mitochondrial trafficking to the IS was significantly faster than the speed of spontaneously migrating mitochondria in unstimulated cells (Fig. 7C). Inhibition of P2Y11 receptor signaling with the antagonist NF340 or the PKA inhibitor H89 impaired the directed movement of mitochondria to the IS, prevented the increase in mitochondrial motility, and abolished mitochondrial Ca^{2+} uptake during IS formation (Fig. 7A-D, Video 4). Based on these findings, we conclude that autocrine stimulation of P2Y11 receptors at the back of polarized T cells promotes the translocation of mitochondria to the IS. The accumulation of mitochondria at the IS is needed to continue ATP production that stimulates P2X4 receptors to deliver localized Ca^{2+} influx that sustains mitochondrial metabolism and continued ATP release into the synaptic gap and beyond. The combined actions of P2X4 and P2Y11 receptors thus induce and stabilize an active immune synapse capable of maintaining the metabolic program needed to complete functional T cell responses following antigen stimulation by APCs (Fig. 7E, Video 5).

DISCUSSION

Purinergic signaling regulates many facets of T cell biology, including cell migration, cytokine production, and T cell proliferation.[14-18] We have previously shown that P2X4 receptors are important for the regulation of these functional responses because they facilitate cellular Ca^{2+} influx that fuels mitochondrial metabolism and ATP production.[15,18] Our current study has shown that P2Y11 receptors have a similarly important role in the regulation of T cell activation, namely by redistributing mitochondria to the IS where these organelles cooperate with P2X4 receptors of stimulated T cells to generate the energy needed for subsequent functional T cell responses.

Others and we have previously demonstrated that mitochondria accumulate at the IS during T cell activation.[23-25,35] Now we show that P2Y11 receptors retract from the IS to the back of cells where they promote the trafficking of mitochondria to the IS through a mechanism that involves cAMP/PKA signaling and the microtubule network. Similar mechanisms have been shown to influence mitochondrial dynamics in other mammalian cells.[41,42] For

example, cytosolic cAMP was shown to cross the outer mitochondrial membrane (OMM) in order to activate PKA signaling events within the intermembrane space, which promotes cristae integrity and mitochondrial function.[43,44] Cytosolic cAMP can also activate PKA that is bound to A-kinase anchor proteins (AKAPs) located on the OMM of mitochondria, which results in the phosphorylation of proteins that are involved in mitochondrial fission and in mitochondrial transport through mechanisms that include kinesin and dynein motor proteins.[45-48] Several other studies have shown that cAMP can promote the directional movement of mitochondria along the microtubule network of neurons.[49-52] All these processes are possible candidate mechanisms by which P2Y11 receptors promote the translocation of mitochondria to the IS during CD4 cell stimulation.

In neurons, kinesin and dynein motors promote anterograde and retrograde mitochondrial transport, respectively.[53] Those molecular motors tie mitochondria to microtubules via adaptor proteins, such as TRAK1, TRAK2, as well as Miro proteins that attach to the OMM.[37,54,55] Cytosolic Ca^{2+} and cAMP signaling act as stop- and go-signals, respectively, that regulate the attachment of mitochondria to the MT network.[42,56] Although the exact mechanisms have yet to be defined, defects in these cAMP/PKA and Ca^{2+} signaling events that regulate mitochondrial trafficking in neurons are known to contribute to degenerative neurological disorders such as Alzheimer's and Parkinson's diseases.[57,58]

While growing evidence has shed light on the importance of mitochondrial trafficking in neurons, comparatively little information exists about how corresponding processes regulate the functions of T cells. We found that nocodazole impairs the recruitment of mitochondria to the IS of T cells, which is consistent with previous reports that mitochondrial transport in T cells occurs along the MT network.[38,59,60] Our findings demonstrate that P2Y11 receptor-induced cAMP/PKA signaling promotes mitochondrial trafficking to the IS. Thus, P2Y11 receptors strengthen the IS by contributing to the accumulation of mitochondria near P2X4 receptors and panx1 channels at the IS, which intensifies localized ATP production, ATP release, and Ca^{2+} influx and thus sustains cell metabolism in order to provide downstream signaling processes with the necessary ATP to complete T cell activation processes.

Taken together with the results of previous studies, we conclude that ATP release and autocrine purinergic signaling via P2X4 and P2Y11 receptors act in synergy to orchestrate mitochondrial trafficking and metabolism during T cell activation. Initial TCR/CD28 signaling induces a brief burst of ATP release that leads to the stimulation of P2X4 receptors. P2X4 receptors then deliver localized Ca^{2+} influx that ignites mitochondrial metabolism at the IS. P2X4 and P2Y11 receptors have similar affinities for ATP with EC_{50} values in the low micromolar range[61], which suggests that the resulting increase in pericellular ATP following T cell stimulation is sufficient to activate first P2X4 at the IS and then the more distant P2Y11 receptors. P2Y11 receptors are displaced from the IS towards the back of cells where their stimulation promotes the trafficking of mitochondria to the IS. This dual P2X4 and P2Y11 receptor signaling process ultimately consolidates the entire mitochondrial mass at the IS where these mitochondria receive Ca^{2+} delivered by P2X4 receptors to generate the ATP that is needed to complete the T cell activation process (Fig. 7E, Video 5). According to this model, P2X4 receptors are upstream actors that help trigger IS formation and that induce P2Y11 receptor stimulation. While P2Y11 receptors are not

necessary for the accumulation of P2X4 receptors at the IS, P2Y11 receptors are responsible for signal amplification and for stabilizing the IS by directing mitochondria to the IS where these organelles work hand-in-hand with P2X4 receptors to fuel T cell metabolism.

Besides P2X4 and P2Y11 receptors, T cells also express P2X7 receptors that are the most extensively studied purinergic receptor subtype of immune cells.[62] In contrast to P2X4 and P2Y11 receptors, P2X7 receptors have a comparatively low affinity for ATP with an EC_{50} value in the millimolar range[61]. P2X7 receptors also remain uniformly distributed across the cell membrane during IS formation and T cell activation.[18] This suggests that the role of the P2X7 receptor is primarily that of a sensor for paracrine ATP that allows T cells to respond to high extracellular ATP environments such as those generated by inflammatory conditions and at tumor sites. Further studies are needed to define how such paracrine ATP input signals affect the P2X7, P2X4, and P2Y11 receptor signaling circuitry that regulates T cell functions. Moreover, similar roles of adenosine receptors, particularly of the $G\alpha_s$ protein-coupled A2 receptors in T cell regulation remain to be elucidated.

Interestingly, mice and other rodents do not possess the P2Y11 receptor gene.[63] Therefore it is possible that A2a or A2b adenosine receptors, the only other known $G\alpha_s$ protein-coupled purinergic receptors capable of inducing cAMP/PKA signaling, must substitute for the role of the missing P2Y11 receptors in rodent cells. This could have important implications for the interpretation of results obtained with rodent models of inflammation and immune disease.

Our experiments have shown that disruption of any of the complex purinergic signaling processes that govern T cell responses results in significant loss of T cell function. These findings have important clinical implications, for example in settings of excessive extracellular ATP accumulation. Extracellular ATP can, for example, accumulate in response to tissue damage and inflammation, which overwhelms the autocrine P2X4 and P2Y11 receptor signaling mechanisms we have found to regulate T cell functions. Dysregulated mitochondrial trafficking, impaired IS signaling, and distorted cell metabolism under such conditions render T cells unable to recognize and launch adaptive immune responses to infections.[28] Indeed, impaired T cell function and exacerbated extracellular ATP accumulation are hallmarks of sepsis and cancer.[3,64,65] Based on our findings, it seems likely that disrupted P2Y11 and P2X4 receptor signaling is an underlying cause and a major contributor to such clinical conditions. In support of this notion, single nucleotide polymorphisms of the human P2Y11 receptor gene have been shown to cause inflammatory disorders that are associated with acute myocardial infarction and narcolepsy.[66-68] These clinical indications further support our conclusion that P2Y11 receptors, like their P2X4 receptor partners, are important regulators of T cell function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AKAP	A-kinase anchor proteins
APC	antigen-presenting cell
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
GPCR	G protein coupled receptor
HPLC	high performance liquid chromatography
ICAM1	intercellular adhesion molecule 1
IS	immune synapse
LAT	linker for activation of T cells
LFA-1	lymphocyte function-associated antigen 1
MT	microtubule
NA	numerical aperture
OMM	outer mitochondrial membrane
panx1	pannexin 1
PBMCs	peripheral blood mononuclear cells
PKA	protein kinase A
pMHC	peptide-loaded major histocompatibility complex
SLP76	SH2 domain-containing leukocyte protein of 76 kDa
TCR	T cell receptor
TRAK	trafficking kinesin protein

REFERENCES

1. Taruno A ATP release channels. Int J Mol Sci. 2018;19(3).pii:E808. [PubMed: 29534490]
2. Lazarowski ER. Vesicular and conductive mechanisms of nucleotide release. Purinergic Signal. 2012;8:359–373. [PubMed: 22528679]
3. Idzko M, Ferrari D, Eltzschig HK. Nucleotide signalling during inflammation. Nature. 2014;509:310–317. [PubMed: 24828189]

4. Burnstock G, Fredholm BB, North RA, Verkhratsky A. The birth and postnatal development of purinergic signalling. Acta Physiol (Oxf). 2010;199:93–147. [PubMed: 20345419]
5. Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev. 1998;50:413–492. [PubMed: 9755289]
6. North RA. Molecular physiology of P2X receptors. Physiol Rev. 2002;82:1013–1067. [PubMed: 12270951]
7. Abbracchio MP, Burnstock G, Boeynaems JM, et al. International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. Pharmacol Rev. 2006;58:281–341. [PubMed: 16968944]
8. Burnstock G Purinergic nerves. Pharmacol Rev. 1972;24:509–581. [PubMed: 4404211]
9. Junger WG. Immune cell regulation by autocrine purinergic signalling. Nat Rev Immunol. 2011;11:201–212. [PubMed: 21331080]
10. Di Virgilio F, Vuerich M. Purinergic signaling in the immune system. Auton Neurosci. 2015;191:117–123. [PubMed: 25979766]
11. Cekic C, Linden J. Purinergic regulation of the immune system. Nat Rev Immunol. 2016;16:177–192. [PubMed: 26922909]
12. Antonioli L, Blandizzi C, Pacher P, Haskó G. The Purinergic System as a Pharmacological Target for the Treatment of Immune-Mediated Inflammatory Diseases. Pharmacol Rev. 2019;7:345–382.
13. Geginat J, Paroni M, Maglie S, et al. Plasticity of human CD4 T cell subsets. Front Immunol. 2014;5:630. [PubMed: 25566245]
14. Wang CM, Ploia C, Anselmi F, Sarukhan A, Viola A. Adenosine triphosphate acts as a paracrine signaling molecule to reduce the motility of T cells. EMBO J. 2014;33:1354–1364. [PubMed: 24843045]
15. Ledderose C, Liu K, Kondo Y, et al. Purinergic P2X4 receptors and mitochondrial ATP production regulate T cell migration. J Clin Invest. 2018;128:3583–3594. [PubMed: 29894310]
16. Schenk U, Westendorf AM, Radaelli E, et al. Purinergic control of T cell activation by ATP released through pannexin-1 hemichannels. Sci Signal. 2008;1:ra66.
17. Yip L, Woehrle T, Corriden R, et al. Autocrine regulation of T-cell activation by ATP release and P2X7 receptors. FASEB J. 2009;23:1685–1693. [PubMed: 19211924]
18. Woehrle T, Yip L, Elkhal A, et al. Pannexin-1 hemichannel-mediated ATP release together with P2X1 and P2X4 receptors regulate T-cell activation at the immune synapse. Blood 2010;116:3475–3484. [PubMed: 20660288]
19. Atarashi K, Nishimura J, Shima T, et al. ATP drives lamina propria T(H)17 cell differentiation. Nature. 2008;455:808–812. [PubMed: 18716618]
20. Schenk U, Frascoli M, Proietti M, et al. ATP inhibits the generation and function of regulatory T cells through the activation of purinergic P2X receptors. Sci Signal. 2011;4:ra12. [PubMed: 21364186]
21. Dustin ML. T-cell activation through immunological synapses and kinapses. Immunol Rev. 2008;221:77–89. [PubMed: 18275476]
22. Dustin ML, Choudhuri K. Signaling and polarized communication across the T cell immunological synapse. Annu Rev Cell Dev Biol. 2016;32:303–325. [PubMed: 27501450]
23. Quintana A, Schwindling C, Wenning AS, et al. T cell activation requires mitochondrial translocation to the immunological synapse. Proc Natl Acad Sci USA 2007;104:14418–14423. [PubMed: 17726106]
24. Baixauli F, Martín-Cófreces NB, Morlino G, et al. The mitochondrial fission factor dynamin-related protein 1 modulates T-cell receptor signalling at the immune synapse. EMBO J. 2011;30:1238–1250. [PubMed: 21326213]
25. Ledderose C, Bao Y, Lidicky M, et al. Mitochondria are gate-keepers of T cell function by producing the ATP that drives purinergic signaling. J Biol Chem. 2014;289:25936–25945. [PubMed: 25070895]
26. Moore DJ, Chambers JK, Wahlin JP, et al. Expression pattern of human P2Y receptor subtypes: a quantitative reverse transcription-polymerase chain reaction study. Biochim Biophys Acta. 2001;1521:107–119. [PubMed: 11690642]

27. Communi D, Robaye B, Boeynaems JM. Pharmacological characterization of the human P2Y11 receptor. Br J Pharmacol. 1999;128:1199–206. [PubMed: 10578132]
28. Sueyoshi K, Ledderose C, Shen Y, Lee AH, Shapiro NI, Junger WG. Lipopolysaccharide suppresses T cells by generating extracellular ATP that impairs their mitochondrial function via P2Y11 receptors. J Biol Chem. 2019;294:6283–6293. [PubMed: 30787105]
29. Mühleder S, Fuchs C, Basílio J, et al. Purinergic P2Y(2) receptors modulate endothelial sprouting. Cell Mol Life Sci. 2019 doi:10.1007/s00018-019-03213-2.
30. Wu J, Liu L, Matsuda T, et al. Improved orange and red Ca^{2}± indicators and photophysical considerations for optogenetic applications. ACS Chem Neurosci. 2013;4:963–972. [PubMed: 23452507]
31. Ledderose C, Woehrle T, Ledderose S, et al. Cutting off the power: inhibition of leukemia cell growth by pausing basal ATP release and P2X receptor signaling? Purinergic Signal. 2016;12:439–451. [PubMed: 27020575]
32. Trebak M, Kinet JP. Calcium signalling in T cells. Nat Rev Immunol. 2019;19:154–169. [PubMed: 30622345]
33. Woehrle T, Ledderose C, Rink J, Slubowski C, Junger WG. Autocrine stimulation of P2Y1 receptors is part of the purinergic signaling mechanism that regulates T cell activation. Purinergic Signal. 2019;15:127–137. [PubMed: 30919205]
34. Glancy B, Balaban RS. Role of mitochondrial Ca^{2+} in the regulation of cellular energetics. Biochemistry. 2012;51:2959–2973. [PubMed: 22443365]
35. Quintana A, Pasche M, Junker C, et al. Calcium microdomains at the immunological synapse: how ORAI channels, mitochondria and calcium pumps generate local calcium signals for efficient T-cell activation. EMBO J. 2011;30:3895–3912. [PubMed: 21847095]
36. Schwarz TL. Mitochondrial trafficking in neurons. Cold Spring Harb Perspect Biol. 2013;5(6).pii:a011304. [PubMed: 23732472]
37. López-Doménech G, Covill-Cooke C, Ivankovic D, et al. Miro proteins coordinate microtubule- and actin-dependent mitochondrial transport and distribution. EMBO J. 2018;37:321–336. [PubMed: 29311115]
38. Morlino G, Barreiro O, Baixauli F, et al. Miro-1 links mitochondria and microtubule Dynein motors to control lymphocyte migration and polarity. Mol Cell Biol. 2014;34:1412–1426. [PubMed: 24492963]
39. Martín-Cófreces NB, Sánchez-Madrid F. Sailing to and docking at the immune synapse: role of tubulin dynamics and molecular motors. Front Immunol. 2018;9:1174. [PubMed: 29910809]
40. Martín-Cófreces NB, Robles-Valero J, Cabrero JR, et al. MTOC translocation modulates IS formation and controls sustained T cell signaling. J Cell Biol. 2008;182:951–962. [PubMed: 18779373]
41. Zhang F, Zhang L, Qi Y, Xu H. Mitochondrial cAMP signaling. Cell Mol Life Sci. 2016;73:4577–4590. [PubMed: 27233501]
42. Di Benedetto G, Gerbino A, Lefkimmiatis K. Shaping mitochondrial dynamics: The role of cAMP signalling. Biochem Biophys Res Commun. 2018;500:65–74. [PubMed: 28501614]
43. Means CK, Lygren B, Langeberg LK, et al. An entirely specific type I A-kinase anchoring protein that can sequester two molecules of protein kinase A at mitochondria. Proc Natl Acad Sci USA. 2011;108:E1227–E1235. [PubMed: 22084075]
44. Darshi M, Mendiola VL, Mackey MR, et al. ChChd3, an inner mitochondrial membrane protein, is essential for maintaining crista integrity and mitochondrial function. J Biol Chem. 2011;286:2918–2932. [PubMed: 21081504]
45. Lefkimmiatis K, Leronni D, Hofer AM. The inner and outer compartments of mitochondria are sites of distinct cAMP/PKA signaling dynamics. J Cell Biol. 2013;202:453–462. [PubMed: 23897891]
46. Sato-Yoshitake R, Yorifuji H, Inagaki M, Hirokawa N. 1992. The phosphorylation of kinesin regulates its binding to synaptic vesicles. J Biol Chem. 1992;267:23930–23936. [PubMed: 1429730]
47. Scherer J, Yi J, Vallee RB. PKA-dependent dynein switching from lysosomes to adenovirus: a novel form of host-virus competition. J Cell Biol. 2014;205:163–177. [PubMed: 24778311]

48. Cribbs JT, Strack S. Reversible phosphorylation of Drp1 by cyclic AMP-dependent protein kinase and calcineurin regulates mitochondrial fission and cell death. EMBO Rep. 2007;8:939–944. [PubMed: 17721437]
49. Müller M, Mironov SL, Ivannikov MV, Schmidt J, Richter DW. Mitochondrial organization and motility probed by two-photon microscopy in cultured mouse brainstem neurons. Exp Cell Res. 2005;303:114–127. [PubMed: 15572032]
50. Ogawa F, Murphy LC, Malavasi EL, et al. NDE1 and GSK3β associate with TRAK1 and regulate axonal mitochondrial motility: identification of cyclic AMP as a novel modulator of axonal mitochondrial trafficking. ACS Chem Neurosci. 2016;7:553–564. [PubMed: 26815013]
51. Das Banerjee T, Dagda RY, Dagda M, et al. PINK1 regulates mitochondrial trafficking in dendrites of cortical neurons through mitochondrial PKA. J Neurochem. 2017;142:545–559. [PubMed: 28556983]
52. Vagnoni A, Bullock SL. A cAMP/PKA/kinesin-1 axis promotes the axonal transport of mitochondria in aging drosophila neurons. Curr Biol. 2018;28:1265–1272. [PubMed: 29606421]
53. Barnhart EL. Mechanics of mitochondrial motility in neurons. Curr Opin Cell Biol. 2016;38:90–99. [PubMed: 26986984]
54. van Spronsen M, Mikhaylova M, Lipka J, et al. TRAK/Milton motor-adaptor proteins steer mitochondrial trafficking to axons and dendrites. Neuron. 2013;77:485–502. [PubMed: 23395375]
55. MacAskill AF, Kittler JT. Control of mitochondrial transport and localization in neurons. Trends Cell Biol. 2010;20:102–112. [PubMed: 20006503]
56. Wang X, Schwarz TL. The mechanism of Ca2+ -dependent regulation of kinesin-mediated mitochondrial motility. Cell. 2009;136:163–174. [PubMed: 19135897]
57. Dagda RK, Das Banerjee T. Role of protein kinase A in regulating mitochondrial function and neuronal development: implications to neurodegenerative diseases. Rev Neurosci. 2015;26:359–370. [PubMed: 25741943]
58. Ould Amer Y, Hebert-Chatelain E. Mitochondrial cAMP-PKA signaling: What do we really know? Biochim Biophys Acta Bioenerg. 2018;1859:868–877. [PubMed: 29694829]
59. Campello S, Lacalle RA, Bettella M, Mañes S, Scorrano L, Viola A. Orchestration of lymphocyte chemotaxis by mitochondrial dynamics. J Exp Med. 2006;203:2879–2886. [PubMed: 17145957]
60. Contento RL, Campello S, Trovato AE, Magrini E, Anselmi F, Viola A. Adhesion shapes T cells for prompt and sustained T-cell receptor signalling. EMBO J. 2010;29:4035–4047. [PubMed: 20953162]
61. Jacobson KA, Jarvis MF, Williams M. Purine and pyrimidine (P2) receptors as drug targets. J Med Chem. 2002;45:4057–4093. [PubMed: 12213051]
62. Di Virgilio F, Dal Ben D, Sarti AC, Giuliani AL, Falzoni S. The P2X7 receptor in infection and inflammation. Immunity. 2017;47:15–31. [PubMed: 28723547]
63. Kennedy C P2Y(11) Receptors: Properties, distribution and functions. Adv Exp Med Biol. 2017;1051:107–122. [PubMed: 29134605]
64. Hotchkiss RS, Monneret G, Payen D. Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach. Lancet Infect. Dis 2013;13:260–268. [PubMed: 23427891]
65. Di Virgilio F, Sarti AC, Falzoni S, De Marchi E, Adinolfi E. Extracellular ATP and P2 purinergic signalling in the tumour microenvironment. Nat Rev Cancer. 2018;18:601–618. [PubMed: 30006588]
66. Amisten S, Melander O, Wihlborg AK, Berglund G, Erlinge D. Increased risk of acute myocardial infarction and elevated levels of C-reactive protein in carriers of the Thr-87 variant of the ATP receptor P2Y11. Eur Heart J. 2007;28:13–18. [PubMed: 17135283]
67. Kornum BR, Kawashima M, Faraco F, et al. Common variants in P2RY11 are associated with narcolepsy. Nat. Genet. 2011;43: 66–71. [PubMed: 21170044]
68. Han F, Faraco J, Dong XS, et al. Genome wide analysis of narcolepsy in China implicates novel immune loci and reveals changes in association prior to versus after the 2009 H1N1 influenza pandemic. PLoS Genet. 2013;9:e1003880. [PubMed: 24204295]

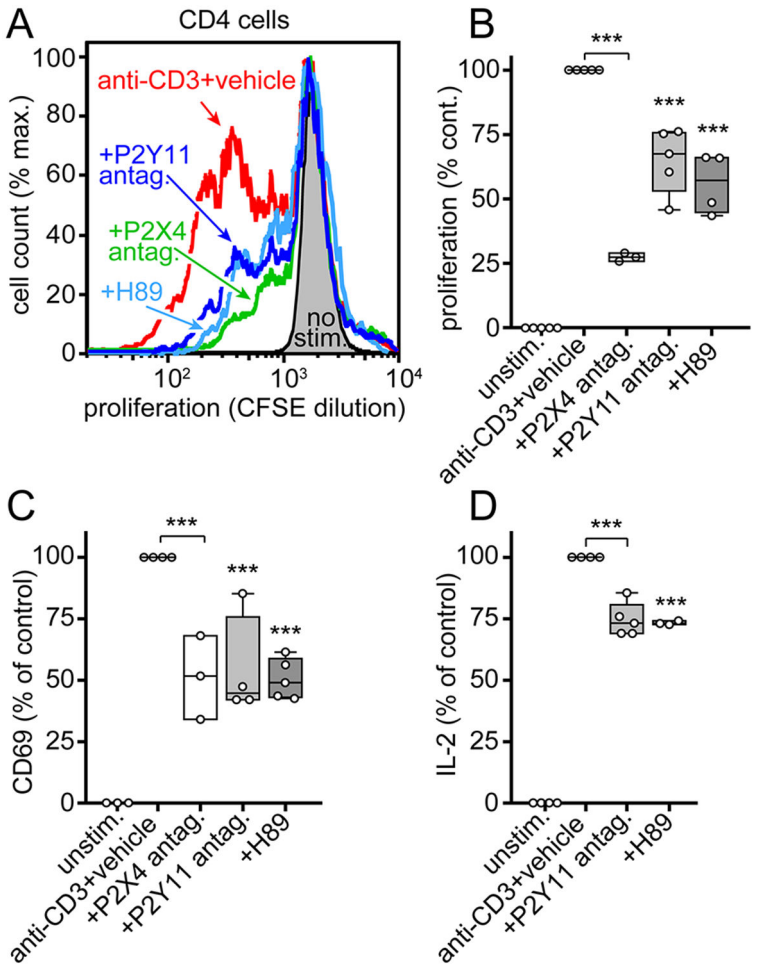

Figure 1. Autocrine signaling via P2Y11 receptors is needed for T cell activation.

(**A-B**) Human PBMCs were labeled with CFSE, treated for 10 min with P2X4 receptor antagonist (5-BDBD; 10 μM), P2Y11 receptor antagonist (NF340; 10 μM), PKA inhibitor (H89; 5 μM), or vehicle control (0.1% DMSO) and then stimulated with anti-CD3 antibodies (0.25 μg/ml). Proliferation of CD4 cells was determined 72 h later using flow cytometry. Representative histograms (**A**) and results from 3-5 independent experiments performed in duplicate with cells from different donors (**B**) are shown. Data in **B** show the fraction of CD4 cells that proliferated under the different conditions relative to cell proliferation under control conditions. The percentage of spontaneously proliferating CD4 cells (2.3 ± 1.7%) was set to 0% and the percentage of cells stimulated in the presence of vehicle control (59.4 ± 7.3%) was set to 100% in order to be able to compare results obtained with cells from different donors; ***P<0.001 vs. stimulated controls (one-way ANOVA). (**C**) Human PBMCs were stimulated with anti-CD3 antibodies in the presence or absence of 5-BDBD, NF340, H89, or vehicle control at the concentrations mentioned above and CD69 expression of CD4 cells was analyzed after 4 h using flow cytometry. Data from 3-5 separate experiments performed in duplicate are shown; ***P<0.001 vs. stimulated control (one-way ANOVA). (**D**) PBMCs were treated as described in panel **C** and IL-2 concentrations in

the supernatants were measured 6 h after cell stimulation. Data are from 3-5 separate experiments performed in duplicate; ***P<0.001 vs. stimulated control (one-way ANOVA).

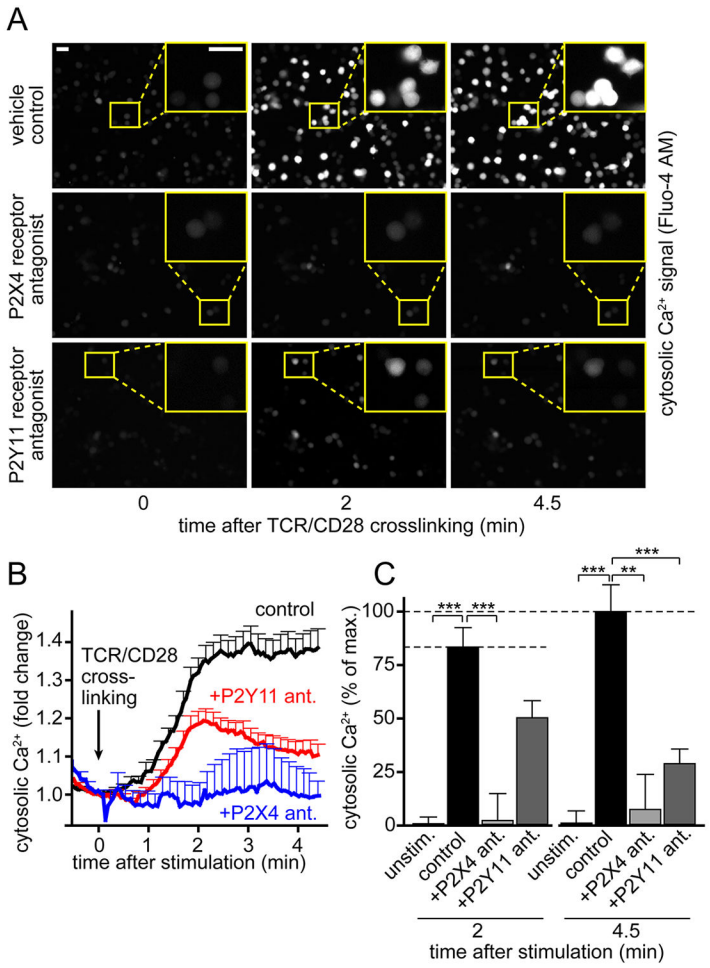

Figure 2. P2X4 and P2Y11 receptors are both needed to sustain Ca^{2+} signaling in response to T cell stimulation.

(**A-C**) Human CD4 cells were labeled with Fluo-4 AM and treated for 10 min with P2X4 receptor antagonist (5-BDBD; 10 μM), P2Y11 receptor antagonist (NF340; 10 μM), or vehicle control (0.1% DMSO). Then anti-CD3 and anti-CD28 antibodies were added, followed by TCR/CD28 crosslinking with anti-mouse IgG antibodies after 30 s (time point = 0 min). Cytosolic Ca^{2+} levels were monitored by fluorescence microscopy. Representative images (**A**), fluorescence traces (**B**), and relative Ca^{2+} responses (**C**) assessed 0, 2, or 4.5 min after TCR/CD28 crosslinking are shown. Data are mean values ± SEM of individual cells (n≥42) derived from at least 3 independent experiments; ** P<0.01, *** P<0.001 vs. stimulated control (Kruskal-Wallis test). Scale bars: 15 μm.

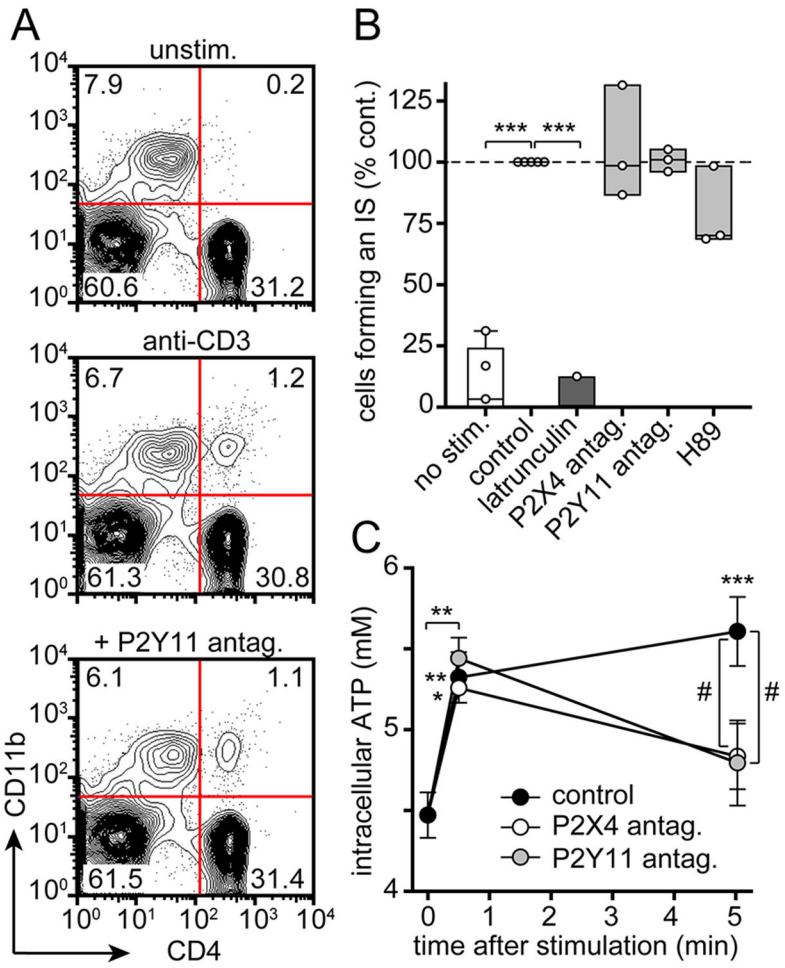

Figure 3. T cells need P2X4 and P2Y11 receptors to sustain cell metabolism following immune synapse formation.

(**A-B**) Human PBMCs were labeled with anti-CD4 and anti-CD11b antibodies and pretreated for 10 min with actin polymerization inhibitor (latrunculin B; 10 μg/ml), P2X4 receptor antagonist (5-BDBD; 10 μM), P2Y11 receptor antagonist (NF340; 10 μM), PKA inhibitor (H89; 5 μM), or with vehicle control (0.1% DMSO). Then, cells were stimulated or not (unstim.) with anti-CD3 antibodies (0.25 μg/ml) and allowed to form monocyte/CD4 cell conjugates for 1 h. These conjugates were identified with flow cytometry by assessing double-positive cell duplets. Representative plots are shown in **A**. The percentages of CD4+ T cells that formed an immune synapse with CD11b+ monocytes are shown in **B**. Data are from 3-5 separate experiments performed in triplicate; ***P<0.001 vs. stimulated control (Kruskal-Wallis test). (**C**) Jurkat cells were treated for 30 min with 5-BDBD, NF340, or vehicle control as described above, stimulated with anti-CD3/CD28 antibody-coated microbeads, and intracellular ATP levels were determined at the indicated time points after cell stimulation. Data are means ± SEM, n=6; *P<0.05, **P<0.05, ***P<0.001 vs. unstimulated cells (one-way ANOVA); #P<0.05 vs. stimulated control (one-way ANOVA).

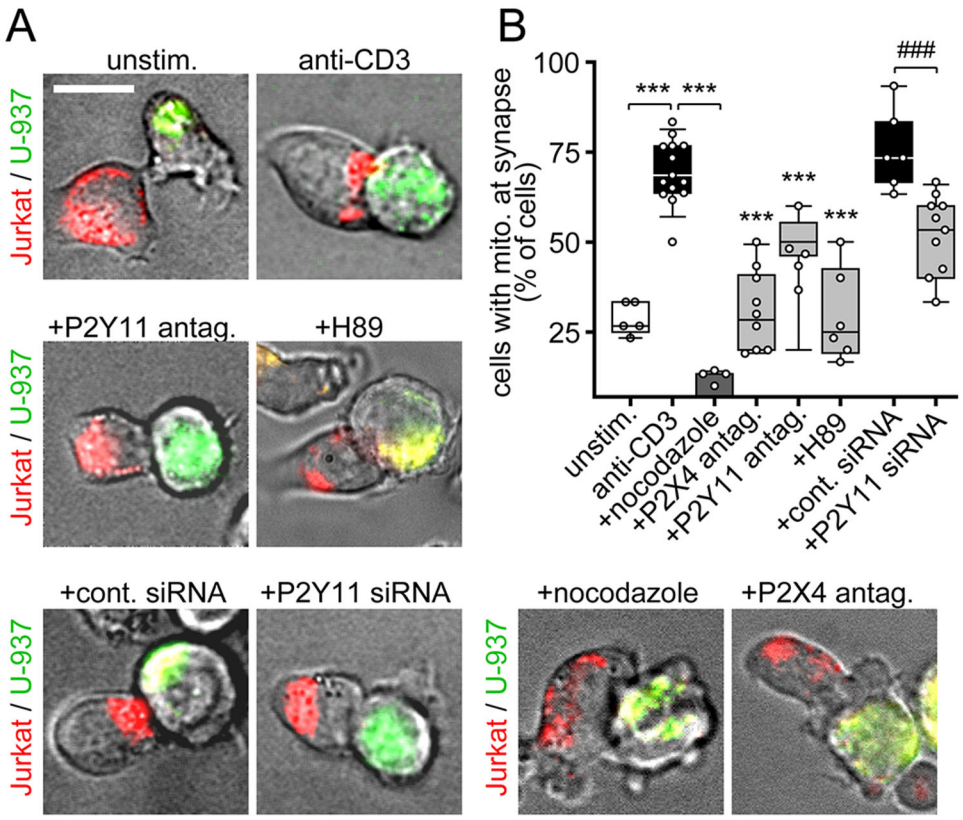

Figure 4. P2Y11 receptor signaling and microtubules promote mitochondrial trafficking to the immune synapse.

(**A-B**) Jurkat T cells labeled with MitoTracker Red CM-H2Xros and U-937 monocytic cells labeled with MitoTracker Green FM were mixed (1:1) and treated for 20 min with an inhibitor of microtubule polymerization (nocodazole; 5 μM), with P2X4 receptor antagonist (5-BDBD; 10 μM), P2Y11 receptor antagonist (NF340; 10 μM), or with a PKA inhibitor (H89; 5 μM). In some cases, Jurkat cells were treated with P2Y11 receptor siRNA or non-targeting control siRNA. Then immune synapse formation was induced by stimulating cells with anti-CD3 antibodies (0.25 μg/ml) and after 15 min the accumulation of mitochondria at the cell-to-cell contact sites between Jurkat cells and U-937 cells was analyzed. (**A**) Representative images acquired with a 40x objective; scale bar: 10 μm. (**B**) Percentage of Jurkat cells with mitochondrial accumulation at the contact site with U-937 monocytic cells. Data shown are from different experiments (n=5-15) each comprising at least 10 separate conjugates; ***P<0.001 vs. stimulated control (one-way ANOVA); ###P<0.001 (t test).

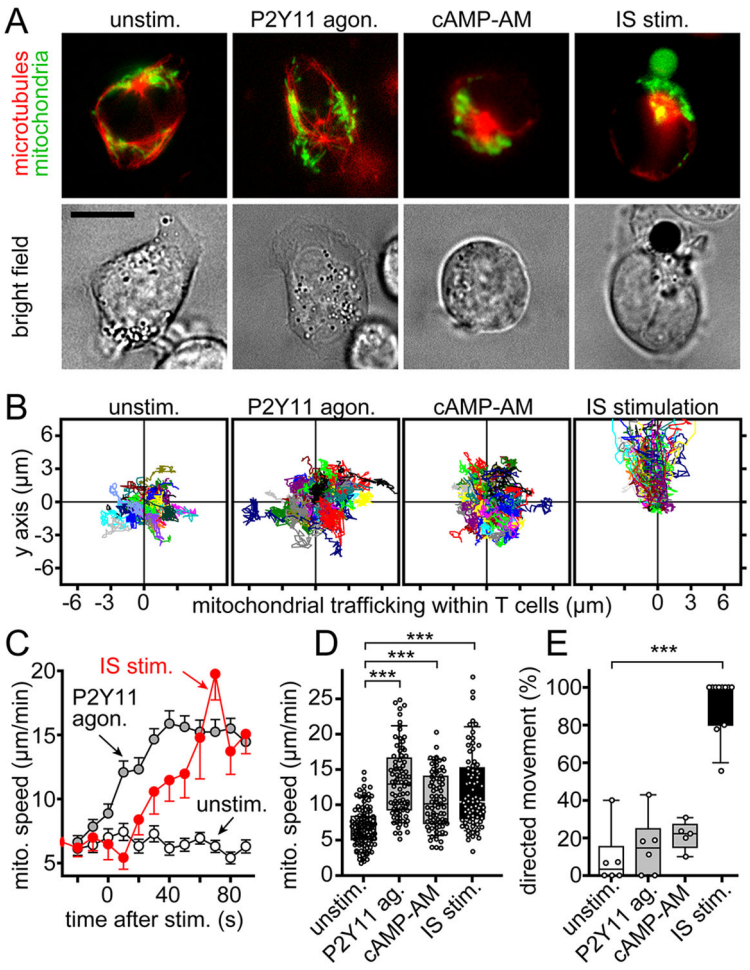

Figure 5. P2Y11 receptors and cAMP signaling augment mitochondrial motility within T cells.

(**A**) Jurkat cells labeled with MitoTracker Red CM-H2Xros and Tubulin Tracker Deep Red were allowed to attach to fibronectin-coated coverslip dishes. Then, cells were treated for 20 min with P2Y11 receptor agonist (NF546; 10 µM) or cell permeable cAMP (cAMP-AM; 1 µM) or they were stimulated with anti-CD3/CD28 antibody-coated microbeads. Mitochondrial motility within cells was imaged using fluorescence microscopy. Images are representative of at least 20 cells analyzed in 3 independent experiments. Note that microbeads shown here in green pseudocolor actually emitted red autofluorescence when imaged using the red TRITC channel. Scale bar: 10 µm. (**B**) Jurkat cells expressing the mitochondrial Ca^{2+} sensor mito-CAR-Geco were treated for 20 min with NF546, cAMP-AM, or stimulated with anti-CD3/CD28 antibody-coated microbeads. Mitochondrial movements within cells were tracked for 3 min and traces of individual mitochondria (n≥50, derived from at least 5 independent experiments) were aligned with their origins at position x=y=0. The position of the bead (IS) in stimulated cells was assigned the coordinate x=0, y=7. (**C**) Mitochondrial trafficking in mito-CAR-Geco-expressing Jurkat cells in response to stimulation at time point t=0 s with either NF546, IS-inducing microbeads, or vehicle control was analyzed as described in **B** (means ± SEM, n=30-50 mitochondria derived from n≥4 separate experiments). (**D-E**) Cells were treated as described in **B** and

mitochondrial speed (**D**) and the directionality of mitochondrial movements towards the IS (**E**) were calculated. Mitochondria were considered to show directed movement to the IS if their migration paths did not deviate by more than 30° from a straight path towards beads (at coordinate x=0, y=7). Data show values obtained with different mitochondria (n=81-113) that were assayed in separate experiments (n=5-11) performed on different days; ***P<0.001 vs. unstimulated controls; Kruskal-Wallis test (**D**) or one-way ANOVA (**E**).

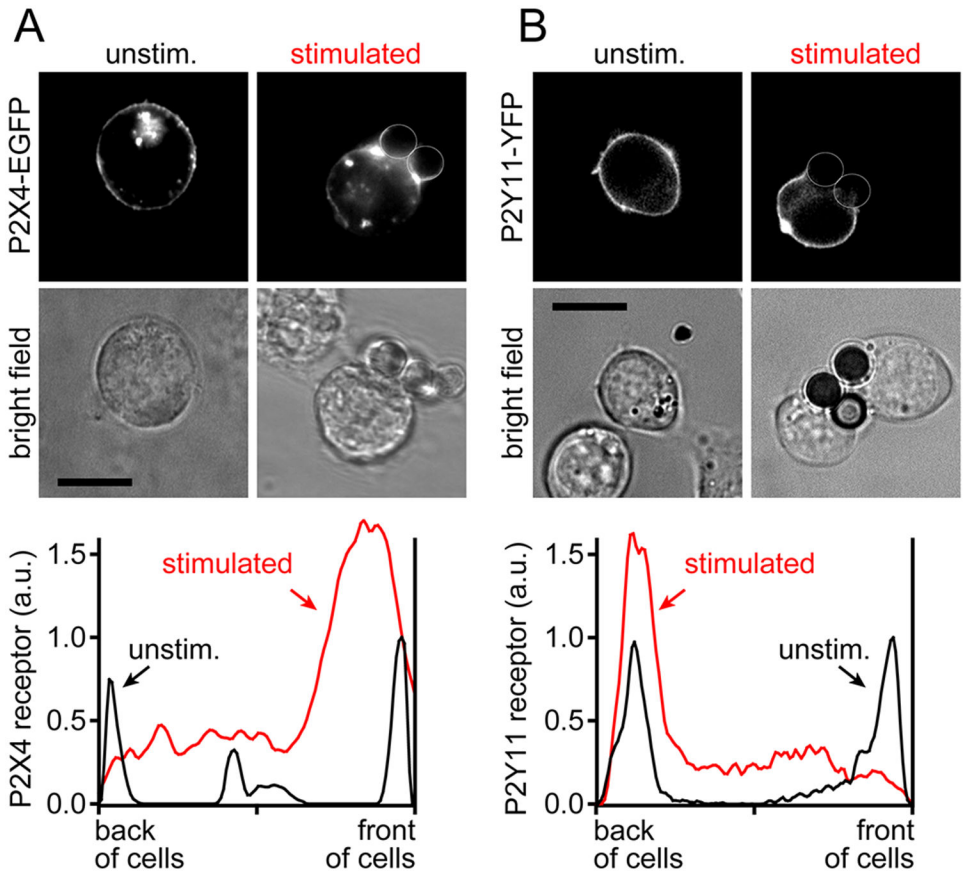

Figure 6. P2Y11 receptors are displaced from the IS, while P2X4 receptors accumulate at the IS.

(**A-B**) Fluorescence and bright field images showing the distribution of EGFP-tagged P2X4 (**A**) and YFP-tagged P2Y11 receptors (**B**) in Jurkat cells before and after stimulation with anti-CD3/CD28 antibody-coated microbeads. Line profiles of averaged fluorescence values summed over the cell area are displayed in both panels below. The images shown are representative of separate experiments (n=5) with similar results. Images were acquired with a 100x oil objective, NA 1.4; scale bars: 10 μm.

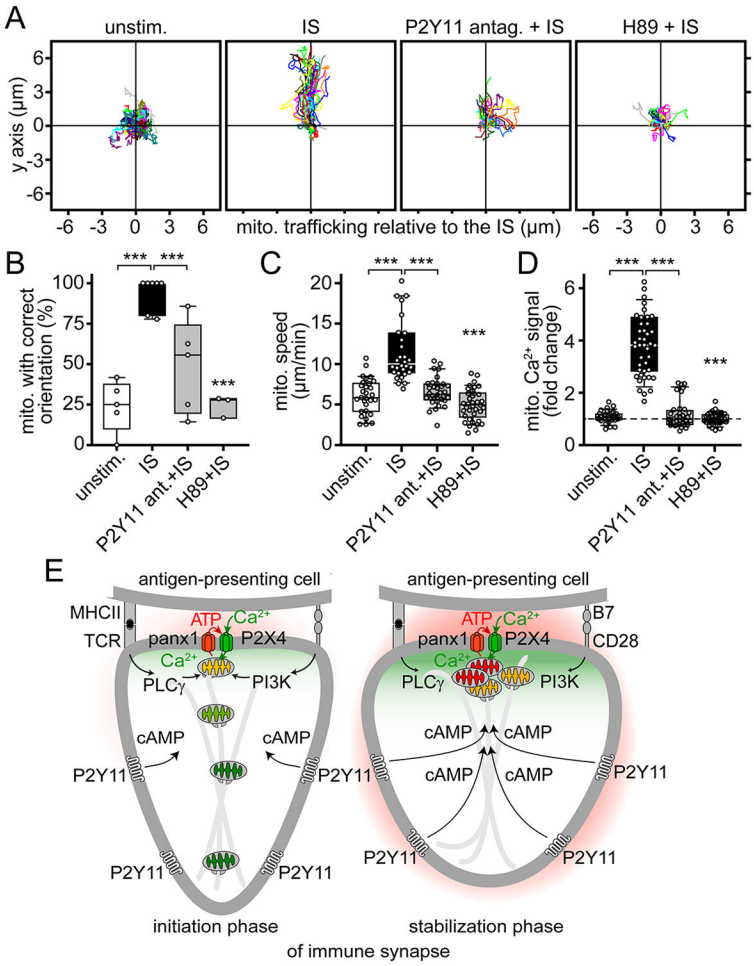

Figure 7. P2Y11 receptors drive mitochondria to the immune synapse where they sustain cell metabolism.

(**A-D**) Jurkat cells expressing the mitochondrial Ca^{2+} sensor mito-CAR-Geco were treated for 20 min with P2Y11 receptor antagonist (NF340; 10 µM), PKA inhibitor (H89; 5 µM), or vehicle control (0.1% DMSO). Then, cells were stimulated with anti-CD3/CD28 antibody-coated microbeads and the directionality (**A, B**) and speed (**C**) of mitochondrial trafficking within cells was assessed. The maximum change in mitochondrial Ca^{2+} uptake (**D**) over a period of 4.5 min was determined with fluorescence microscopy. Traces of individual mitochondria were aligned with their origins at coordinate x=y=0 as shown in panel **A**. Mitochondria were considered to move in the correct direction if their migration paths did not deviate by more than 30° from a straight line towards the IS (at coordinate x=0, y=7). Data in **B** show the results of independent experiments (n=3-7; indicated by circles) with separate mitochondria (n=6-18) analyzed in each experiment; *** P<0.001 vs. IS-stimulated controls; one-way ANOVA. Data in **C** and **D** show results obtained from different mitochondria (n≥30) assessed in separate experiments (n≥3); *** P<0.001 vs. IS-stimulated control; Kruskal-Wallis test. (**E**) Proposed mechanism by which P2X4 and P2Y11 receptors cooperate to initiate and stabilize functional immune synapses required for T cell activation. Stimulation of T cell receptors (TCR) and CD28 co-receptors by antigen-presenting cells triggers localized ATP release that stimulates adjacent P2X4 receptors. Stimulated P2X4

receptors promote Ca^{2+} influx that causes mitochondrial ATP production and further ATP release by upregulation of P2X4 receptors at the IS. This upregulated ATP response is sufficient to stimulate the more distant P2Y11 receptors that induce cAMP/PKA signaling and promote trafficking of mitochondria along microtubules to the IS. The accumulation of mitochondria, P2X4 receptors, and pannexin1 (panx1) channels stabilizes the IS and sustains the metabolic profile needed to complete T cell activation processes. Differently colored mitochondria represent different degrees of mitochondrial activity ranging from low (green) and medium (orange) to high (red).

Video 1. Translocation of mitochondria to the immune synapse depends on P2Y11 receptors.
Jurkat T cells labeled with MitoTracker Red CM-H2Xros (T cell) and U-937 monocytic cells labeled with MitoTracker Green (APC) were treated for 20 min with microtubule assembly disruptor (nocodazole; 5 μM), P2X4 antagonist (5-BDBD; 10 μM), P2Y11 antagonist (NF340; 10 μM), or PKA inhibitor (H89; 5 μM). In some cases, Jurkat cells were transfected with P2Y11-targeting or control siRNA. Then, cells were stimulated with anti-CD3 antibodies and bright field and fluorescence image sets were captured (frame rate: 6 frames per minute; 40x objective).

Video 2. P2Y11 receptor signaling promotes mitochondrial motility.

Jurkat cells expressing the mitochondrial Ca^{2+} sensor mito-CAR-Geco were treated for 20 min with P2Y11 receptor agonist (NF546; 10 μM), cell-permeable cAMP (cAMP-AM; 1 μM), or stimulated with anti-CD3/CD28 antibody-coated microbeads to induce immune synapse (IS) formation. Mitochondrial movements within cells were imaged with fluorescence microscopy (frame rate: 1 frame per second; 100x objective). Superimposed traces of single mitochondrial migration paths are displayed in the bottom panels.

Video 3. P2Y11 receptors are not required for P2X4 receptor accumulation at the immune synapse.

Jurkat cells expressing EGFP-tagged P2X4 receptors were pretreated with P2Y11 receptor antagonist (NF340; 10 μM) or vehicle control (medium) for 10 min, stimulated with anti-CD3/CD28 antibody-coated microbeads, and immune synapse formation and P2X4 receptor translocation were recorded and analyzed with bright field and fluorescence image pairs (frame rate: 12 frames per minute).

Video 4. P2Y11 receptors drive mitochondria to the immune synapse.

Jurkat cells expressing the mitochondrial Ca^{2+} sensor mito-CAR-Geco were treated for 20 min with P2Y11 receptor antagonist (NF340; 10 μM), PKA inhibitor (H89; 5 μM), or vehicle control (0.1% DMSO). Cells were then stimulated with anti-CD3/CD28 antibody-coated microbeads and bright field and fluorescence image pairs were captured (frame rate: 12 frames per minute; 100x objective). Superimposed traces of single mitochondrial migration paths are displayed in the panels on the right.

Video 5. Animation model of proposed mechanism by which P2X4 and P2Y11 receptors cooperate to form and stabilize the immune synapse during T cell activation.

T cell receptor (TCR) stimulation by antigen presenting cells that display a matching antigen triggers immune synapse (IS) formation. This results in the accumulation of P2X4 receptors and panx1 ATP release channels at the IS. P2X4 receptors amplify an initial burst of ATP release by facilitating Ca^{2+} influx that upregulates mitochondrial metabolism at the IS. The resulting ATP release is now sufficient to activate more distant P2Y11 receptors that are displaced from the IS. These P2Y11 receptors induce cAMP/PKA signaling that promotes the trafficking of mitochondria to the immune synapse. This process stabilizes the IS and supports the sustained ATP production that T cells need for subsequent functional T cell responses.