Mitochondria Are Gate-keepers of T Cell Function by Producing the ATP That Drives Purinergic Signaling*

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Background: Autocrine purinergic signaling regulates T cell function.

Results: We found that mitochondria are the source of ATP that drives these signaling mechanisms.

Conclusion: Mitochondria are the gate-keepers of T cell function that control purinergic signaling and T cell activation.

Significance: These findings suggest that mitochondria could be therapeutic targets to modulate T cell responses.

T cells play a central role in host defense. ATP release and autocrine feedback via purinergic receptors has been shown to regulate T cell function. However, the sources of the ATP that drives this process are not known. We found that stimulation of T cells triggers a spike in cellular ATP production that doubles intracellular ATP levels in <30 s and causes prolonged ATP release into the extracellular space. Cell stimulation triggered rapid mitochondrial Ca2+ uptake, increased oxidative phosphorylation, a drop in mitochondrial membrane potential (∆Ψm), and the accumulation of active mitochondria at the immune synapse of stimulated T cells. Inhibition of mitochondria with CCCP, KCN, or rotenone blocked intracellular ATP production, ATP release, intracellular Ca2+ signaling, induction of the early activation marker CD69, and IL-2 transcription in response to cell stimulation. These findings demonstrate that rapid activation of mitochondrial ATP production fuels the purinergic signaling mechanisms that regulate T cells and define their role in host defense.

Critically ill patients often develop profound immunosuppression and nosocomial infections due to impaired T cell function (1). However, the underlying mechanisms leading to T cell dysfunction have remained unclear (2).

The release of cellular ATP via pannexin-1 channels (panx1)2 and autocrine feedback through purinergic receptors are being recognized as essential steps required for proper T cell activation (3, 4). We found that the release of ATP stimulates several purinergic receptors, including P2X1, P2X4, and P2X7 and that these ATP receptors collectively contribute to the influx of extracellular Ca2+ that is required for IL-2 production and effector functions in response to T cell stimulation (5, 6).

Despite the growing recognition of the importance of ATP release and purinergic signaling in T cell activation, the intracellular sources of the ATP that is released from stimulated T cells are not known. ATP is the main energy carrier that drives virtually all cellular processes. Quiescent T cells rely mainly on the metabolism of glucose, amino acids, and fatty acids for ATP production, which depends on the TCA cycle and oxidative phosphorylation (7, 8). T cell stimulation induces a metabolic switch toward aerobic glycolysis, which allows rapidly dividing T cells to meet their extensive energetic and biosynthetic demands. Because of the central role of glycolysis in proliferating T cells, mitochondrial ATP production has been considered to have a secondary, if any role in T cell activation (8–10). However, recent evidence suggests that mitochondria may have a more profound role in T cell activation. For example, mitochondria have been shown to accumulate at the immune synapse (IS) that T cells form with antigen-presenting cells (APC). Mitochondria are thought to be necessary in order to regulate Ca2+ homeostasis and to generate reactive oxygen species that modulate signaling pathways downstream of the TCR/CD28 co-receptor (11–13).

Here we show that mitochondria play another critical role in T cell activation, namely the production of intracellular ATP that is released from stimulated cells in order to fuel the purinergic signaling mechanisms that regulate T cell activation at the IS. Our findings indicate that mitochondria are gate-keepers of T cell activation that coordinate Ca2+ homeostasis with autocrine purinergic signaling processes to orchestrate the complex choreography of T cell activation events required for a successful immune response.

EXPERIMENTAL PROCEDURES

Materials—Dynabeads (Invitrogen Dynal AS, Oslo, Norway) or polystyrene particles (3.0–3.9 μm, Spherotech, Lake Forest, IL) coated with goat anti-mouse IgG antibodies were labeled with mouse anti-human CD3 and anti-human CD28 antibodies.
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(BD Biosciences, San Jose, CA) and used for T cell stimulation. Mouse anti-human CD69-FITC antibodies were purchased from BD Biosciences. MitoTracker Red CM-H$_2$XRos, Fluo-4 AM, Rhod-2 AM, JC-1, and dihydrorhodamine 123 (DHR) were purchased from Molecular Probes (Grand Island, NY). All other reagents were of the highest grade and from Sigma-Aldrich if not stated otherwise.

Cell Culture and Transfection—Jurkat cells (clone E6–1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 medium (ATCC) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Invitrogen) at 37 °C in 5% CO$_2$. Jurkat cells transiently expressing the mitochondrial Ca$^{2+}$ indicator mito-CAR-GECO1 (14) were generated by electroporation with CMV-mito-CAR-GECO1 (Addgene plasmid number 46022; Addgene, Cambridge, MA) using a Neon transfection system (Invitrogen). Peripheral blood mononuclear cells (PBMC) were isolated from the heparinized venous blood of healthy volunteers using Ficoll-Paque Plus (GE Healthcare, Pittsburgh, PA) and CD4$^+$ T cells were purified from PBMC with anti-CD4 magnetic beads (Miltenyi Biotec, San Diego, CA) according to the manufacturer’s instructions.

Mice—The Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center (BIDMC) approved the use of animals. C57BL/6 wild-type control mice were from the Jackson Laboratory (Bar Harbor, ME) and panx1 heterozygous knock-out mice were from the Knock-out Mouse Project (KOMP) Repository at the University of California Davis (Davis, CA), backcrossed with C57BL/6 mice, and maintained as homozygous knock-out mouse colony. Animals were euthanized, spleens harvested, red blood cells removed by Ficoll density-gradient centrifugation, and remaining splenocyte preparations cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. For T cell stimulation, cells (1 × 10$^6$/ml) were transferred to 48-well plates precoated with anti-mouse anti-CD3/CD28 antibodies or uncoated wells (control) and incubated for 4 h at 37 °C in a tissue culture incubator. IL-2 mRNA expression was determined by qPCR.

Patients—Eligible patients were patients presenting to the Emergency Department of the Beth Israel Deaconess Medical Center (BIDMC) aged 18 years or older who were clinically diagnosed with severe sepsis or septic shock (classified according to the criteria of the ACCP/SCCM consensus conference, 15). Leukocyte-rich plasma was obtained at the day of diagnosis immediately after blood draw by spontaneous sedimentation (1 × g) over Ficoll-Paque Plus for 30 min at 37 °C. All studies involving human subjects were approved by the Institutional Review Board of BIDMC, and written informed consent was obtained.

ATP Release and Intracellular ATP Production—Jurkat cells or purified CD4$^+$ T cells (5 × 10$^5$) were treated with 2-deoxy-D-glucose (2-DG; 20 mM), CCCP (10 μM) or oligomycin (10 μM), or stimulated with anti-CD3/anti-CD28 antibody-coated beads (2 beads/cell) for the indicated periods of time, and intracellular ATP concentrations or ATP in the cell-free supernatant were determined using an ATP Bioluminescence HS II assay kit (Roche, Indianapolis, IN) or high performance liquid chromatography (HPLC) of samples after etheno-derivatization of adenine compounds as previously described (16). For the assessment of intracellular ATP concentrations, cells were snap-frozen in liquid nitrogen, sonicated in the presence of 0.4 M perchloric acid, and analyzed by HPLC.

Assessment of ATP Release at the Cell Surface and of Intracellular ATP Bursts using the ATP Probes 2–2Zn(II) and 1–2Zn(II)—The membrane-bound ATP probe 2–2Zn(II) and the intracellular ATP probe 1–2Zn(II) were a generous gift of Drs. Kurishita and Hamachi (Kyoto University). Binding of ATP and other polyphosphate compounds to these probes causes a shift in their fluorescence properties. 2–2Zn(II) has a lipid anchor residue that binds to the cell membrane and can thus be used to assess ATP release at the cell surface (17). For real-time imaging of ATP release Jurkat cells were seeded on fibronectin-coated glass bottom dishes and stained with 500 nM 2–2Zn(II) in Hank’s balanced salt solution (HBSS) for 5 min at 37 °C. Fluorescence live-cell imaging was performed with an inverted Leica DMI6000B microscope (Leica, Wetzlar, Germany) equipped with a temperature controlled stage incubator (Harvard Apparatus, Holliston, MA) and a Spot Boost EMCCD BT 2100 camera (Diagnostic Instruments Inc., Sterling Heights, MI). Fluorescence images were taken through 100× (NA 1.3) or 63× (NA 1.4) oil objectives (Leica) using an YFP-2427A filter set (Semrock, Rochester, NY) and μManager software that was developed in Ron Vale’s laboratory at UCSF and with funding from the National Institutes of Health, NIH Grant R01-EB007187. The ATP cell surface concentration was estimated by comparison of fluorescence signals obtained with ATP standards (1–100 μM).

ATP release during synapse formation was also assessed by flow cytometry (BD FACSCalibur, Becton Dickinson, San Jose, CA). Jurkat cells were stained with 2–2Zn(II) as described above and stimulated with anti-CD3/anti-CD28 antibody-coated polystyrene beads, which display negligible autofluorescence. Cell/bead aggregates were identified by their characteristic forward and side scattering properties and the percentage of 2–2Zn(II)$^+$ cells releasing high amounts of ATP was determined where cells were considered 2–2Zn(II)$^+$ whose mean fluorescence intensity was higher than that of 95% of unstimulated control cells.

For imaging of intracellular ATP firing, cells were loaded with 100 μM of the lipid anchor-free ATP probe, 1–2Zn(II), in the presence of 1% DMSO. After incubating for 30 min, cells were washed, stimulated with beads, and studied by fluorescence microscopy.

Imaging of Mitochondria—Translocation of mitochondria to the IS was monitored by bright field and fluorescence time-lapse imaging of Jurkat cells loaded with MitoTracker Red CM-H$_2$XRos (100 nM) for 20 min at 37 °C and stimulated with anti-CD3/CD28 coated beads. To study the association between mitochondrial localization and ATP release, Jurkat cells were loaded with MitoTracker Red CM-H$_2$XRos, washed, and stained with 2–2Zn(II). Three-dimensional imaging was done on a Ultraview Vox Spinning Disk Confocal microscope (PerkinElmer, Boston, MA) equipped with a Hamamatsu C9100–13 camera (IDDR Imaging Core, Boston Children’s Hospital, Boston, MA) using Velocity Software.
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Mitochondrial Membrane Potential ($\Delta \psi_m$) and ROS Production—The ratiometric mitochondrial probe JC-1 was used to monitor $\Delta \psi_m$ after cell stimulation. Cells were incubated with JC-1 (1 μg/ml) for 20 min at 37 °C in RPMI 1640 medium, washed, stimulated with anti-CD3/CD28-coated beads and analyzed by FACS at the indicated times after stimulation. The green (FL1) and red (FL2) fluorescence channels were used to detect the monomer (green) and J-aggregate (red) forms of JC-1.

Dihydrorhodamine 123 (DHR) was used to study mitochondrial ROS production. DHR is cell-permeable and non-fluorescent whereas its oxidation product, rhodamine 123, is positively charged and fluorescent and binds selectively to the inner mitochondrial membrane of living cells (18). Cells were stained with DHR (2 μM, 20 min) in RPMI 1640 medium, washed, stimulated with anti-CD3/CD28 antibody-coated beads and analyzed using live-cell fluorescence microscopy or flow cytometry as described above.

Intracellular $\text{Ca}^{2+}$ Measurement—Jurkat cells were loaded with the $\text{Ca}^{2+}$ indicator Fluo-4 AM (4 μM) to assess $\text{Ca}^{2+}$ signaling before and after treatment (10 min) with CCCP (1 μM), the panx1 inhibitor CBX (20 μM), or the P2 receptor antagonist suramin (200 μM). Cells were stimulated with beads and changes in intracellular $\text{Ca}^{2+}$ levels were measured by flow cytometry. Rhod-2 AM (2 μM) was used to monitor changes in mitochondrial $\text{Ca}^{2+}$ concentrations. Mitochondrial localization of the dye was verified by microscopy. Confocal bright field and fluorescence time-lapse images were captured using a Leica DMi6000B microscope equipped with a spinning disk (CARVII, Becton Dickinson). The mitochondrial $\text{Ca}^{2+}$ influx following bead stimulation after pretreatment (10 min) or not (control) with suramin (200 μM) was measured with a Hitachi F-4500 Fluorescence Spectrophotometer equipped with a stirred cuvette (Hitachi). For some experiments, Jurkat cells expressing the $\text{Ca}^{2+}$ biosensor mito-CAR-GECO1 were used to study changes in mitochondrial $\text{Ca}^{2+}$ concentrations. Time-lapse images were recorded 4–6 h after transfection using an inverted fluorescence microscope.

IL-2 mRNA and CD69 Expression—Cells (5 × 10^6) were pretreated for 10 min with CCCP (1 μM), potassium cyanide (KCN, 500 μM), rotenone (1 μM), suramin (200 μM), or culture medium (control) followed by stimulation with anti-CD3/CD28 antibody-coated beads for 30 min at 37 °C. Expression of the early T cell activation marker CD69 was analyzed by flow cytometry. IL-2 mRNA expression was determined by quantitative real-time PCR. RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) and converted to cDNA (QuantiTect reverse transcription kit, Qiagen) according to the manufacturer’s instructions. Real-time PCR was performed on a Mastercycler ep realplex instrument (Eppendorf, Hamburg, Germany) using iQ™ SYBR® Green supermix (Bio-Rad) and QuantiTect primer assays (Qiagen). IL-2 expression was normalized to $\beta$-actin, and the comparative $C_t$ method was used for relative quantification of gene expression.

Statistical Analyses—Unless otherwise stated, data are expressed as mean values ± standard deviation (S.D.) of n ≥ 3 independent experiments. Statistical analyses were done using Student’s t test and differences were considered statistically significant at $p < 0.05$.

RESULTS

T Cell Stimulation Triggers Rapid Intracellular ATP Production—We have previously found that stimulated T cells release ATP and that purinergic receptors have an essential role in the early signaling cascade that results in T cell activation. While it is now well established that panx1 channels are one important mechanism by which T cells can release ATP (3, 5, 19, 20), it has remained unclear what triggers the rapid opening of panx1 channels in T cells and how ATP is generated prior to its release. In order to begin addressing these open questions, we examined the timing of ATP release in response to T cell stimulation. Jurkat T cells and primary human CD4+ T cells were stimulated with anti-CD3/CD28 antibody-coated beads and extracellular ATP concentrations were measured with HPLC analysis after different times. We found that both, Jurkat cells and CD4+ T cells very rapidly released ATP with extracellular ATP concentrations reaching half-maximal levels in less than 30 s after cell stimulation (Fig. 1, A and B). Interestingly, Jurkat cells released over 10-times more ATP than CD4+ T cells.

To search for the cellular sources of the released ATP, we assessed the intracellular concentrations of ATP, ADP, AMP, and adenosine before and after cell stimulation. To our surprise, we found that intracellular ATP levels did not drop in response to ATP release. Instead, we found a rapid increase in intracellular ATP levels that peaked in less than 30 s after cell stimulation. Intracellular ATP levels increased by up to 100%
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A and B, resting Jurkat cells derive their ATP mainly from glycolysis and primary CD4+ T cells mainly from mitochondrial oxidative phosphorylation. Jurkat cells (A) or CD4+ T cells (B) were treated with vehicle control (black), 2-DG (20 mM, blue) to block glycolysis or CCCP (10 μM, red) or oligomycin (10 μM, green) to inhibit mitochondrial ATP production for the indicated times, and intracellular ATP levels were measured with a luciferase bioluminescence assay. Data show means ± S.D. of n = 4 independent experiments; *, p < 0.05 versus control. C, Jurkat cells were treated with CCCP (1 μM) for 10 min, stimulated with anti-CD3/CD28 coated beads for 30 s, and iATP was assessed by HPLC. Data represent means ± S.D. of n = 3 independent experiments; *, p < 0.05 versus vehicle control. D, Jurkat cells (D) were treated with the indicated concentrations of CCCP and CD4+ T cells (E) were treated with CCCP (1 μM), rotenone (1 μM) or vehicle control for 10 min and ATP release into the supernatant was measured with a bioluminescence assay after stimulating cells for 30 s with anti-CD3/CD28-coated beads. Data represent means ± S.D. (n = 3); *, p < 0.05 versus control. 

FIGURE 2. Mitochondria generate the ATP that is released by stimulated T cells. A and B, resting Jurkat cells derive their ATP mainly from glycolysis and primary CD4+ T cells mainly from mitochondrial oxidative phosphorylation. Jurkat cells (A) or CD4+ T cells (B) were treated with vehicle control (black), 2-DG (20 mM, blue) to block glycolysis or CCCP (10 μM, red) or oligomycin (10 μM, green) to inhibit mitochondrial ATP production for the indicated times, and intracellular ATP (iATP) levels were measured with a luciferase bioluminescence assay. Data show means ± S.D. of n = 4 independent experiments; *, p < 0.05 versus control. C, Jurkat cells were treated with CCCP (1 μM) for 10 min, stimulated with anti-CD3/CD28 coated beads for 30 s, and iATP was assessed by HPLC. Data represent means ± S.D. of n = 3 independent experiments; *, p < 0.05 versus control. D, Jurkat cells (D) were treated with the indicated concentrations of CCCP and CD4+ T cells (E) were treated with CCCP (1 μM), rotenone (1 μM) or vehicle control for 10 min and ATP release into the supernatant was measured with a bioluminescence assay after stimulating cells for 30 s with anti-CD3/CD28-coated beads. Data represent means ± S.D. (n = 3); *, p < 0.05 versus control.

and remained elevated for at least 5 min. Interestingly, this increase in intracellular ATP concentrations was not paralleled by a decrease in intracellular concentrations of ADP, AMP, or adenosine (Fig. 1, C and D). Instead, we found that the concentrations of these ATP precursors barely changed during T cell activation. This suggests that ATP production during T cell activation requires additional mechanisms besides the phosphorylation of low energy adenine precursors that are present in the cells. These additional mechanisms may involve nucleoside scavenging pathways, creatine kinases, and de novo synthesis pathways that form precursors for ATP production (21, 22).

Taken together our results indicate that T cell stimulation triggers virtually instantaneous ATP release, which is fueled by equally rapid processes that increase intracellular ATP concentrations.
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beads carrying antibodies to stimulate TCR/CD28 receptors triggered instantaneous ATP release from membrane sites associated with the beads (Fig. 3A, supplemental Movie S1). ATP release intensified at the IS during T cell activation, reaching local ATP concentrations of an estimated 80 μM at the cell surface (Fig. 3B, supplemental Fig. S1C). We also used 2–2Zn(II) in conjunction with flow cytometry and found that a significantly higher percentage of cells engaged with beads stained positive for ATP release compared with cells that were not attached to beads (Fig. 3C). The percentage of T cells with elevated ATP release peaked immediately after cell stimulation and remained elevated over a period of at least 60 min (Fig. 3D). To further analyze how focal TCR/CD28 stimulation influences ATP production and release we stimulated cells with beads coated with anti-CD3/CD28 antibodies or with soluble anti-CD3 antibodies. We then measured ATP concentrations in the supernatant and inside the cells. We found that both ATP release (Fig. 3E) and the increase in intracellular ATP (Fig. 3F) were significantly higher when cells were stimulated with beads coated with anti-CD3/CD28 antibodies compared with cell stimulation with soluble anti-CD3 antibodies alone.

Mitochondrial Translocation Facilitates ATP Release at the IS—The results above suggest that the cellular redistribution of mitochondria contributes to localized ATP release at the IS. To test this notion, we labeled Jurkat cells with MitoTracker dye and monitored mitochondrial translocation during IS formation. Mitochondria rapidly redistributed to the site of cell stimulation and accumulated at the IS that the cells formed with beads (Fig. 4A, supplemental Movie S2). Dual staining of Jurkat cells with 2–2Zn(II) and MitoTracker dye revealed hotspots of ATP release at the IS formation and mitochondrial translocation are actin cytoskeleton-dependent processes (12, 25).

IS formation and mitochondrial translocation are actin cytoskeleton-dependent processes (12, 25). Latrunculin B, an inhibitor of actin polymerization, markedly reduced the release of ATP from cells stimulated with beads. This suppression by
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LATRUNCULIN B was in the same range as that caused by the gap channel inhibitor carbamoylcholine (CBX; Fig. 4C). In contrast to CBX, latrunculin B also reduced the increase in intracellular ATP levels after cell stimulation (Fig. 4D). Taken together, these findings suggest that mitochondrial translocation has an important role in shaping the spatiotemporal patterns of ATP release at the IS during T cell activation.

**T Cell Stimulation Is Associated with a Rapid Drop in Mitochondrial Membrane Potential \( \Delta \psi_m \)—**The findings above suggest that ATP release is not merely a function of panx1 channel opening, but rather associated with rapid activation of mitochondrial ATP production. Mitochondrial ATP production is driven by oxidative phosphorylation, which requires the maintenance of an electrochemical gradient across the inner mitochondrial membrane that is sustained by the electron transport chain of mitochondria. The mitochondrial membrane potential \( \Delta \psi_m \) is therefore an important parameter related to the capacity of cells to produce ATP with the help of their mitochondria.

To study the functional response of mitochondria during T cell stimulation, we investigated whether T cell stimulation affects \( \Delta \psi_m \). Cells were stained with JC-1 and analyzed by flow cytometry. JC-1 is a fluorescent mitochondrial probe that allows monitoring of \( \Delta \psi_m \) based on changes in its green and red fluorescence properties. Stimulation with anti-CD3/CD28 antibody coated beads caused a rapid increase in green and a concomitant decrease in red JC-1 fluorescence, suggesting a drop in \( \Delta \psi_m \) (Fig. 5A). These changes occurred virtually instantaneously after cell stimulation (Fig. 5, B and C). The ratio of red versus green fluorescence, a measure for \( \Delta \psi_m \), dropped rapidly within 1 min after the stimulation of primary CD4+ T cells (Fig. 5B) and Jurkat cells (Fig. 5C) and remained below the values of unstimulated control cells for at least 30 min. CCCP, which uncouples oxidative phosphorylation, was used as a control and caused a further drop and complete dissipation of \( \Delta \psi_m \). The decrease in \( \Delta \psi_m \) following T cell stimulation suggests intense mitochondrial activity and ATP production due to heavy ion flux and ATP release across the mitochondrial membrane. As with stimulated T cells, firing neurons are also known to show a drop in \( \Delta \psi_m \) during bursts of heavy mitochondrial activity, which involves vigorous mitochondrial Ca2+ uptake and ATP release (26). Taken together with these findings, our results suggest that mitochondrial depolarization and massive mitochondrial ATP production are characteristics not only of synaptic activity in neurons but also in T cells that generate ATP for release into the IS.

**T Cell Stimulation Triggers Mitochondrial ROS Production—**The decrease in \( \Delta \psi_m \) in stimulated T cells described above is consistent with increased mitochondrial activity and ATP production (26). Enhanced TCA cycle activity that energizes the electron transport chain not only increases the capability of mitochondria to produce ATP but also to form reactive oxygen species (ROS) as byproducts of ATP production. In T cells, ROS are almost exclusively generated in mitochondria and ROS formation can therefore serve as a read-out of mitochondrial activity. Using the cell-permeable fluorescent probe dihydrorhodamine 123 (DHR) for the monitoring of ROS formation, we found that T cell stimulation increased mitochondrial ROS production (Fig. 6). When studying ROS formation with flow cytometry, we found that mitochondrial activity peaked instantly after cell stimulation. ROS production remained elevated above baseline for at least 20 min after stimulation of CD4+ T cells or Jurkat cells with beads (Fig. 6A). This rapid triggering of mitochondrial activity was also seen in live-cell imaging experiments, where we observed marked ROS production in CD4+ T cells and Jurkat cells after their initial contact and stimulation with beads (Fig. 6B, supplemental Movie S4).

**Mitochondrial Ca2+ Influx Following T Cell Stimulation Triggers Intracellular ATP Production—**Activation of oxidative phosphorylation requires mitochondrial Ca2+ uptake (27–29). Using the mitochondrial Ca2+ probe Rhod-2, we found that T cell stimulation with beads caused rapid mitochondrial Ca2+ uptake in primary CD4+ T cells (Fig. 7A, supplemental Movie S5) and in Jurkat cells (Fig. 7B, supplemental Movie S6). Treatment with latrunculin B or CCCP to prevent mitochondrial translocation or to induce disruption of \( \Delta \psi_m \) respectively, completely abolished mitochondrial Ca2+ influx in Jurkat cells expressing the mitochondria-targeting Ca2+ biosensor mito-CAR-GECO1 (Fig. 7, C and D, supplemental Movie S7). Suramin had no effect in these experiments, indicating that mitochondrial Ca2+ uptake occurs independently of P2 receptors (Fig. 7E). To assess concomitant intracellular ATP production, we used an intracellular ATP probe, 1–2Zn(II) that was also developed in the laboratory of Prof. Hamachi and is structurally identical to...
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2–2Zn(II), except that 1–2Zn(II) lacks the membrane anchor residue and can be taken up by cells (30). Using this probe, we found that mitochondrial firing in response to cell stimulation resulted in rapid bursts of intracellular ATP release, apparently by the release of ATP from activated mitochondria (supplemental Movie S8). Taken together, these findings demonstrate that T cell stimulation triggers rapid mitochondrial ATP production accompanied by bursts in mitochondrial Ca\(^{2+}\) uptake and oxidative phosphorylation, as well as by a sudden drop in \(\Delta\Psi_m\).

Mitochondrial ATP Production Regulates Cellular Ca\(^{2+}\) Influx in Response to T Cell Stimulation—Proper T cell activation is known to require an initial increase in intracellular Ca\(^{2+}\) concentrations followed by a sustained elevation of cytosolic Ca\(^{2+}\) levels due to the influx of Ca\(^{2+}\) from the extracellular space (31). We have previously shown that P2X receptors contribute to the Ca\(^{2+}\) influx in stimulated T cells (4, 5). To test whether mitochondrial activation is required for Ca\(^{2+}\) influx, we loaded cells with the cytosolic Ca\(^{2+}\) indicator Fluo-4 AM and studied how inhibition of mitochondrial ATP production affects Ca\(^{2+}\) signaling in the cytosol of stimulated T cells. We found that inhibition of mitochondrial ATP production with CCCP blocked sustained Ca\(^{2+}\) signaling. A similar effect was seen by inhibition of ATP release with the panx1 inhibitor CBX.

Inhibition of P2 receptors with suramin further blocked cellular Ca\(^{2+}\) signaling (Fig. 7F), but did not alter mitochondrial Ca\(^{2+}\) uptake (Fig. 7E). This indicates that mitochondrial ATP production is required for the sustained phase of Ca\(^{2+}\) signaling, and that mitochondrial ATP and purinergic signaling are needed to sustain Ca\(^{2+}\) levels in stimulated T cells.

Mitochondrial ATP Production Is Required to Elicit Functional T Cell Responses—The findings described above indicate that mitochondrial ATP firing is an essential step in T cell activation. The importance of this process for proper T cell activation is supported by the findings that inhibition of mitochondria with CCCP blocked the expression of the early T cell
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Our findings indicate that mitochondria have a central role in T cell activation by producing the ATP that is required for purinergic signaling and maintaining Ca^{2+} homeostasis. Mitochondria thus occupy a key regulatory position in which they integrate the inside-out (purinergic) and outside-in (Ca^{2+}) signaling networks that control T cell activation. This finding is interesting given the fact that glycolysis, and not mitochondrial ATP production, is the predominant mechanism by which activated T cells generate the bulk of their ATP demand (7–9). The function of mitochondria during T cell activation shifts from ATP production to the production of TCA intermediates that are required for biosynthesis (9, 32). However, emerging evidence points to a more complex involvement of mitochondria in the T cell activation process (33, 34). Our current findings support this notion. We found that mitochondria are not only rapidly activated but that they also produce large amounts of ATP immediately after TCR/CD28 stimulation. Mitochondrial ATP production is a highly dynamic process that can rapidly up-regulate intracellular ATP to levels that match regional demands, specifically at the IS. Mitochondria are responsible for these rapid increases in intracellular ATP levels and for the production of the ATP that is delivered into the extracellular space during cell stimulation, where ATP propels the purinergic signaling events that trigger T cell activation. However, our data also show that the rapid increase in cellular ATP production during T cell activation requires additional mechanisms that provide the ADP that mitochondria phosphorylate to ATP. It is likely that purine salvage pathways are involved in these mechanisms (22). Several immune dysfunctions, including SCID, are caused by deficiencies of key enzymes involved in purine salvage pathways (35–37). However, it is also possible that de novo purine synthesis and creatine kinases are involved in the formation of ADP that can be phosphorylated and converted to ATP in mitochondria (21). Further studies are needed to delineate these mechanisms.

Our novel finding that mitochondria provide ATP for auto-crime purinergic signaling at the IS is supported by previous reports that suggest a direct involvement of mitochondria in T cell activation. For example, mitochondria were shown to accumulate at the IS and to regulate store-operated Ca^{2+} signaling by buffering inflowing Ca^{2+} ions, thus preventing termination of Ca^{2+} signaling (11, 12, 38). Our current findings extend this concept by showing that mitochondria can regulate Ca^{2+} signaling at the IS by fueling purinergic signaling mechanisms that facilitate sustained Ca^{2+} influx in a P2X receptor-mediated fashion. Our novel concept that mitochondria pump ATP into the IS extends previous reports that the mitochondrial fission factor DRP-1 positions mitochondria to the peripheral supramolecular activation cluster (pSMAC) of the IS and regulates mitochondrial ATP production near the IS (24).

Several other reports have suggested that mitochondria-derived ROS, the byproducts of mitochondrial ATP production, function as secondary messengers that are necessary for T cell activation (39–41). Recently, Sena et al. reported that ROS spe-
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FIGURE 9. Proposed model of the regulation of Ca\(^{2+}\) and purinergic signaling by mitochondrial firing. TCR/CD28 stimulation triggers Ca\(^{2+}\) release from intracellular stores, resulting in mitochondrial Ca\(^{2+}\) firing and ATP production that feeds autocrine purinergic signaling and prolonged Ca\(^{2+}\) influx via P2X1 and P2X4 receptors at the immune synapse.

cifically generated at complex III of the electron transport chain are required for activation of nuclear factor of activated T cells (NFAT) and subsequent induction of IL-2 and proliferation in CD4\(^{+}\) T cells. The authors also showed that ROS production depends on Ca\(^{2+}\) influx and mitochondrial Ca\(^{2+}\) uptake (13). Taken together with these findings, our results indicate that mitochondrial ROS as well as ATP production have important roles in T cell activation. We found a spike in ATP production that may fuel autocrine purinergic signaling processes that contribute to Ca\(^{2+}\) influx as well as intracellular processes associated with T cell proliferation. Taken together with previous reports, we conclude that mitochondria have at least three different tasks in T cell activation: generating a rapid burst in ATP production that triggers autocrine purinergic signaling, production of ROS that regulate downstream signaling pathways, and regulation of cytosolic Ca\(^{2+}\) homeostasis that sustains T cell responses. T cells are able to respond with remarkable sensitivity and selectivity to the faint antigen-induced signals generated at the IS (42). While it has been proposed that T cells must possess sophisticated amplification mechanisms that help them respond to such weak external signals, the nature of these amplification mechanisms has remained elusive (42). Purinergic signal amplification could explain this enigma. We have previously shown that panx1 channels and probably additional release mechanisms (3, 5, 43). ATP in the IS then stimulates P2X receptors that function as ATP-gated Ca\(^{2+}\) channels and facilitate Ca\(^{2+}\) influx. These inside-out/outside-in feedback loops escalate mitochondrial ATP production, thereby amplifying TCR/CD28 signaling and sustaining Ca\(^{2+}\) influx through P2X receptors.

Impairments of these interconnected signaling processes can inhibit T cell function and cause immunosuppression (44). Critical care patients are particularly prone to such complications, which increases their risk of developing nosocomial infections and sepsis (1, 2). It has long been known that patients with severe trauma or burn injuries are unable to appropriately generate IL-2 in response to T cell stimulation (45, 46) and that Ca\(^{2+}\) homeostasis in T cells is affected (47). We found that mitochondrial activity in lymphocytes of septic patients is impaired, which may be an underlying cause of T cell suppression in these patients. This notion is supported by findings that oxygen consumption of peripheral blood mononuclear cells is lower in sepsis patients than in patients without sepsis (48, 49) and that the cellular ATP content of circulating cells is lower and lactate levels are higher in sepsis patients compared with patients without sepsis (50).

All these findings point toward mitochondria as important regulators of T cell function and as sentinels that help protect the host against infections and sepsis. Mitochondria share many similarities with their close bacterial relatives that include the rickettsiae, which are obligate intracellular aerobic bacteria and cause devastating diseases such as typhus (51). In contrast to these pathogens, mitochondria have evolved to protect their mammalian host from microbial invaders and their own bacterial relatives.

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REFERENCES

1. Hotchkiss, R. S., Monneret, G., and Payen, D. (2013) Immunosuppression in sepsis: a novel understanding of the disorder and a new therapeutic approach. Lancet Infect. Dis. 13, 260–268
2. Hotchkiss, R. S., Monneret, G., and Payen, D. (2013) Sepsis-induced immunosuppression: from cellular dysfunction to immunotherapy. Nat. Rev. Immunol. 13, 862–874
3. Schenk, U., Westendorf, A. M., Radaelli, E., Casati, A., Ferro, M., Fumagalli, M., Verderio, C., Buer, J., Scanziani, E., and Grassi, F. (2008) Purinergic control of T cell activation by ATP released through pannexin-1 hemichannels. Sci. Signal 1, ra6
4. Yip, L., Woehrle, T., Corrider, R., Hirsh, M., Chen, Y., Inoue, Y., Ferrari, Y., Insel, P. A., and Junger, W. G. (2009) Autocrine regulation of T-cell activation by ATP release and P2X7 receptors. FASEB J. 23, 1685–1693
5. Woehrle, T., Yip, L., Elkhah, A., Sumi, Y., Chen, Y., Yao, Y., Insel, P. A., and Junger, W. G. (2010) Pannexin-1 hemichannel-mediated ATP release together with P2X1 and P2X4 receptors regulate T-cell activation at the immune synapse. Blood 116, 3475–3484
6. Junger, W. G. (2011) Immune cell regulation by autocrine purinergic signaling. Nat. Rev. Immunol. 11, 201–212
7. Pearce, E. L. (2010) Metabolism in T cell activation and differentiation. Curr. Opin Immunol. 22, 314–320
8. Fox, C. J., Hammerman, P. S., and Thompson, C. B. (2005) Fuel feeds function: energy metabolism and the T-cell response. Nat. Rev. Immunol. 5, 844–852

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