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Mitochondrial Superoxide Generation Enhances P2X7R-Mediated Loss of Cell Surface CD62L on Naive Human CD4+ T Lymphocytes

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Migration of naive CD4+ T lymphocytes into lymphoid tissue is essential for their activation and subsequent roles in adaptive immunity. The adhesion molecule L-selectin (CD62L), critical for this process, is highly expressed on naive CD4+ T lymphocytes and is downregulated upon T lymphocyte activation. We demonstrate protein expression of P2X7R on naive CD4+ T lymphocytes and show functional channel activity in whole-cell patch clamp recordings. CD62L downregulation occurs rapidly in response to extracellular ATP, a process that is blocked by selective antagonists of P2X7R. This loss of surface CD62L expression was not associated with externalization of phosphatidylserine. While investigating the mechanisms for this process, we revealed that pharmacological modulation of mitochondrial complex I or III, but not inhibition of NADPH oxidase, enhanced P2X7R-dependent CD62L downregulation by increasing ATP potency. Enhanced superoxide generation in the mitochondria of rotenone- and antimycin A–treated cells was observed and may contribute to the enhanced sensitivity of P2X7R to ATP. P2X7R-dependent exposure of phosphatidylserine was also revealed by preincubation with mitochondrial uncouplers prior to ATP treatment. This may present a novel mechanism whereby P2X7R-dependent phosphatidylserine exposure occurs only when cells have enhanced mitochondrial reactive oxygen species generation. The clearance of apoptotic cells may therefore be enhanced by this mechanism which requires functional P2X7R expression. The Journal of Immunology, 2013, 190: 1551–1559.

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Abbreviations used in this article: ADAM, a disintegrin and metalloproteinase domain-containing protein; CD62L, L-selectin; DCF, 2′,7′-dichlorodihydrofluorescein diacetate; MMP, matrix metalloproteinase; PFC, protein kinase C; PS, phosphatidylserine; ROS, reactive oxygen species; SLO, secondary lymphoid organ; SNP, single nucleotide polymorphism.

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tion of FOXO1 from the nucleus, regulates the expression of KLF2 transcription factor-dependent genes including CD62L, as well as CCR7 and S1P1 (27, 28).

The mechanisms coupling P2X7R activation to CD62L down-regulation are unclear and the aim of this study was to investigate established and novel signaling mechanisms in the regulation of CD62L. Previous studies have used RNA and protein techniques as well as dye uptake based assays, exploiting the second permeation state following P2X7R activation, to confirm P2X7R expression. In this study, we show P2X7R functional expression in naive human CD4 + T lymphocytes using whole cell patch clamp electrophysiology as well as P2X7-dependent loss of surface CD62L expression. Remarkably, inhibitors of mitochondrial electron transport significantly enhanced potency of ATP/P2X7R-mediated CD62L downregulation. Phosphatidylserine (PS) exposure was not observed in response to ATP alone; however, pretreatment with rotenone and antimycin A revealed P2X7R-dependent PS externalization. This suggests that modulation of mitochondrial function, which occurs during apoptosis, may promote clearance of apoptotic naive CD4 + T lymphocytes in a P2X7R-dependent manner.

Materials and Methods

Reagents

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich. GM6001 was purchased from Calbiochem (Merck; Darmstadt, Germany). A438079 and AZ11645373 were purchased from Tocris Bioscience (Bristol, U.K.). P2X7R and Erk1/2 Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). MitosoxRed and 2,7'-dichlorodihydrofluorescein diacetate (DCF) were purchased from Invitrogen (Paisley, U.K.). Annexin V/propidium iodide apoptosis kit was purchased from Southern Biotechnology Associates (Atlanta, GA).

Cells

Naive CD4 + T lymphocytes were isolated from the peripheral blood of healthy volunteer donors using a naive CD4 + T cell isolation kit II human (MACS; Miltenyi Biotec). Procedures using human blood were carried out following manufacturer’s guidelines. Isolated naive CD4 + T lymphocytes were cultured in RPMI 1640 medium (supplemented with 10% FCS, 100 U/ml penicillin, and 10 µg/ml streptomycin) and incubated in a 37°C, 5% CO2 incubator.

Immunoblotting

The cell stimulations, cell lysis, and Western blotting were performed as described previously (29). Cells were treated as stated, centrifuged 300 × g for 30 s, and lysed by addition of 100 µl solubilization buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 1 mM sodium vanadate, sodium molybdate, 10 mM sodium fluoride, 40 mM PMSE, 0.7 g/ml pepstatin A, 10 g/ml aprotinin, 10 g/ml leupeptin, and 10 g/ml soybean trypsin inhibitor). The samples were mixed and gently rotated at 4°C for 20 min and then centrifuged at 13,000 × g for 10 min. The supernatant was transferred to fresh tubes and diluted 4:1 with 10% SDS containing 5% sample buffer. Before loading onto the gel, samples were boiled for 5 min at 100°C. The samples were separated by electrophoresis in 10% SDS-PAGE. Proteins were then electrotransferred onto nitrocellulose membrane, blocked in 5% milk, and incubated with anti-P2X7R (H-265) (1/1000 dilution, number SC-25698; Santa Cruz Biotechnology) as primary Ab and anti-rabbit HRP (1/10,000,000 dilution) as secondary Ab. Immune complexes were visualized using ECL Western blotting system (Amersham Biosciences, Little Chalfont, U.K.).

Electrophysiology

Ion currents were measured using the whole-cell patch clamp technique as previously described (30). Borosilicate glass GC150 TF-10 capillaries (Harvard Apparatus, Kent, U.K.) were pulled in two stages using a Narishige PC-10 and then fire-polished with a Narishige MPB30 Microforge to give a filled resistance of between 3 and 5 MΩ. Internal recording solution contained: KCl (147 mM), HEPES (10 mM), EGTA (1 mM) and pH adjusted to 7.4 with KOH. External solution contained: NaCl (147 mM), KCl (2 mM), CaCl2 (2 mM), MgCl2 (1 mM), HEPES (10 mM), and n-glucose (12 mM) and pH adjusted to 7.4 with NaOH. Suspension cells were allowed to settle in the recording bath for at least 20 min. Recordings were performed using voltage clamp at the holding potential of −60 mV with a HEKA EPC10 amplifier using Patchmaster version 2.11 software. All agonists and antagonists were applied using an RSC-200 rapid solution changer (BioLogic, Cliax, France) built from GC100 T-10 glass capillaries (Harvard Apparatus). Antagonists were applied 1 s prior to the subsequent coapplication with the agonist. The extracellular divalent cations Ca2+ and Mg2+ are known to inhibit P2X7R function; therefore, for electrophysiology, unless stated otherwise, agonists and antagonists were applied in external solution without MgCl2 or CaCl2 (31, 32).

CD62L measurement by flow cytometry

For each experimental condition 0.5 × 10^6 naive CD4 + T lymphocytes were treated with vehicle/inhibitors before the addition of ATP at given concentrations. Cells were incubated in a 37°C, 5% CO2 incubator for the indicated times. Cells were washed twice with PBS plus 2% FCS and then stained for 1 h with either isotype control (IgG1k- FITC) or CD62L-FITC on ice for 1 h. After labeling, cells were washed an additional two times and then analyzed using a FACSCanto flow cytometer (BD Biosciences, Oxford, U.K.). Cells were excited at the wavelength 488 nm, and the emission wavelength was recorded at 530/30 nm.

Reactive oxygen species generation detection

Freshly isolated naive CD4 + T lymphocytes were incubated with 10 µM DCF (Invitrogen) in RPMI 1640 medium (without supplements) for 45 min at ambient room temperature protected from light. Cells were washed by centrifugation at 300 × g for 5 min. Cells were resuspended in the same external solution as for electrophysiology 5 min prior to agonist application. Fluorescence was monitored using a multidetection plate reader (Fluostar Optima, BMG Labtech, Aylesbury, U.K.; excitation, 485 nm; emission, 520 nm). Measurements were performed in triplicate per treatment group. Linear regression was performed to determine the rate of reactive oxygen species (ROS) generation.

To measure mitochondrial O2 levels, cells were loaded with 2.5 µM MitoSOX Red (Invitrogen) for 30 min at 37°C protected from light. Cells were then washed by centrifugation at 300 × g for 5 min, treated with agonist for the indicated time in complete RPMI 1640 cell culture medium, washed into PBS, and analyzed immediately using flow cytometry. MitoSOX Red fluorescence was detected at the excitation and emission wavelengths of 488 and 585 nm, respectively.

Measurement of PS externalization

Naive CD4 + T lymphocytes were treated with extracellular ATP at the indicated concentrations in complete RPMI 1640 medium. For each condition, 0.25 × 10^6 cells were washed twice in ice-cold PBS and then resuspended to a concentration of 1 × 10^6 cells/ml in Annexin V binding buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, 2.5 mM CaCl2, and 0.1% BSA). Using an Annexin V/PI apoptosis kit (Southern Biotechnology Associates), allophycocyanin-conjugated Annexin V was then added to each tube, and cells were incubated on ice protected from light for 15 min. Additional Annexin V binding buffer was added, and cells were immediately analyzed by flow cytometry at the excitation and emission wavelengths of 563 and 660 nm, respectively.

Data analysis and statistics

For CD62L expression and MitoSOX Red, flow cytometry was analyzed using FACSDiva software (BD Biosciences, San Jose, CA); a gate was set around naive CD4 + T lymphocytes, and mean fluorescence index was recorded for all cells in this gate. Alternatively, for measurement of PS exposure, a second gate was set containing 1% of unstained cells positive for fluorescence; the percentage of fluorescence positive cells from treatment groups stained with Ab were recorded. Graphs were plotted and concentration/inhibition sigmoidal curves were fitted using Prism 4 (GraphPad, La Jolla, CA); statistical analysis was performed using this software.
Results

P2X7R is expressed in T lymphocytes and functions as an ion channel

To explore further the function of P2X7R expression in human T lymphocytes, we first verified expression on primary human naive CD4+ T lymphocytes freshly isolated from the blood. We also assessed the leukemic T cell line Jurkat as well as the monocyte leukemic cell line THP-1. Cell lysates were immunoblotted with anti-P2X7R Ab to examine the expression of this receptor (Fig. 1A). Specificity of the Ab for P2X7R was confirmed by comparing HEK293 cells transfected with vector only or a P2X7R plasmid.

Having verified protein expression of P2X7R in naive CD4+ T lymphocytes freshly isolated from peripheral human blood, we performed whole-cell patch clamp recordings to evaluate functional ion channel expression. Previous studies have investigated the effect of divalent cations on P2X7R activation (3, 31, 32), and concurrent with this, we demonstrate that naive CD4+ T lymphocytes exhibit a small inward current in response to extracellular application of 5 mM ATP (10 s), which is increased when MgCl2 and CaCl2 were removed from the application buffer (Fig. 1B–D). ATP is reported to act as an agonist for cloned human P2X7R stably expressed in HEK293 cells with an EC50 value of 1.8 and ~0.7 mM, where external electrophysiological solution contained normal or low MgCl2/CaCl2 concentrations, respectively (33, 34), whereas the reported EC50 for ATP interacting with other P2X receptors occurs with much lower values (35). Extracellular ATP evoked ionic currents in a concentration-dependent manner with a response detected with 1 mM ATP, suggesting the activation of P2X7R (Fig. 1E). The sensitivity to a P2X7-selective competitive antagonist (A438079) was investigated to determine the contribution of P2X7R to ATP-induced currents in T lymphocytes. A438079 inhibits activation of human P2X7R expressed in cell lines with an IC50 value of 0.1–0.3 μM measured by calcium influx, large m.w. dye uptake, and IL-1β release (36). In this study, application of 10 μM A438079 significantly inhibited 5 mM ATP evoked currents in naive CD4+ T lymphocytes (n = 5; p < 0.05) (Fig. 1F, 1G) confirming the activation and functional expression of P2X7R.

P2X7R activation causes CD62L downregulation

P2X7R has been shown to couple to CD62L downregulation in both mouse and human T lymphocytes (20). In this study, we further explore the mechanism integrating ATP signaling through P2X7R to CD62L processing. Naive CD4+ T lymphocytes isolated from peripheral human blood show uniform high levels of CD62L surface expression as well as displaying a naive expression pattern of other markers including CCR7 (Supplemental Fig. 1A–C). PMA through activation of PKC is known to cause rapid potent CD62L downregulation (37). Indeed, stimulation of freshly isolated human naive CD4+ T lymphocytes with 100 nM PMA for 30 min induced significant CD62L downregulation. Treatment with 3 mM ATP for 1 h also caused significant CD62L downregulation (Fig. 2A, Supplemental Fig. 1D). The level of ATP-induced CD62L downregulation after a 1-h 3 mM treatment varied significantly between donors, whereas TCR-induced loss was less variable (Supplemental Fig. 1E). ATP-induced CD62L downregulation was rapid with a peak loss after 15 min and sustained low surface expression for up to 6 h (Fig. 2B). We next investigated temporal relationship between ATP treatment and CD62L downregulation (Fig. 2C). Concentration response curves show EC50 values of 877 μM (5-min stimulation) and 888.6 μM (1-h stimulation), suggesting the sustained involvement of P2X7R over time (38). To confirm P2X7R function in this process using available pharmacological tools, we chose two P2X7R antagonists: A438079 (which blocks ATP induced currents in Fig. 1) and a noncompetitive P2X7R antagonist AZ11645373 (Fig. 2D). Published data indicate that AZ11645373 is more potent than A438079 with IC50 values in the range of 5–90 nM at human P2X7R expressed in HEK293 cells and in THP-1 monocytes (33). Pretreatment for 30 min with both antagonists inhibited CD62L downregulation in response to a 1-h 3 mM ATP treatment with IC50 values of 2.25 μM (A438079) and 1.35 μM (AZ11645373). In naive CD4+ T lymphocytes these antagonists act with an order of magnitude less potency than figures published for HEK293 cells transfected with human P2X7R (33, 36).

We initially used small molecule kinase inhibitors to look for signaling molecules involved in ATP induced CD62L loss; how-
ever, we report that PI3K, MEK-Erk1/2, and PKC signaling are not required for this process (Supplemental Fig. 2). PI3K and MEK-Erk1/2 signaling have been shown to be dispensable for ATP-induced processing of CD27 in mouse lymphocytes (39). We reasoned that, because P2X7R activation leads to calcium influx (40) and CD62L processing can be activated by raising intracellular free Ca2+ (22), ATP-induced CD62L downregulation might be dependent on influx of Ca2+ through P2X7R. Although elevation of cytosolic Ca2+ by thapsigargin (1–100 μM) caused significant downregulation of CD62L, the absence or presence of calcium had no significant impact on ATP-induced CD62L downregulation (n = 3; p = 0.9736) (Supplemental Fig. 3). This is consistent with previous studies that have shown Ca2+ influx is not necessary for ATP-induced CD62L downregulation (19). These observations indicate that, although increases in cytosolic Ca2+ levels can cause CD62L downregulation, ATP-induced loss of CD62L in naive CD4+ T lymphocytes occurs via a calcium-independent mechanism.

ADAM17 is the principal proteinase responsible for CD62L cleavage in response to a number of activating factors, but recently evidence has suggested that P2X7R-mediated CD62L downregulation also occurs through ADAM10 activation (22). When naive CD4+ T lymphocytes were pretreated with GM6001 (100 μM), a broad spectrum matrix metalloproteinase (MMP) inhibitor, significant inhibition of CD62L downregulation in response to 1-h 3 mM ATP was observed (n = 3; p < 0.05) (Fig. 2E).

ATP couples to reactive oxygen species generation

P2X7R activation can lead to generation of intracellular ROS, and this drives biochemical processes within cells (41). However, little is known about the role of P2X7R in ROS generation in T lymphocytes. ADAM17 can be activated by ROS through oxidation of cysteine motifs (42), and we therefore hypothesized that ROS generation in response to ATP could activate ADAM17 and subsequently lead to CD62L processing. Treatment of naive CD4+ T lymphocytes with ATP caused an increase in the rate of ROS generation compared with vehicle alone (Fig. 3A). With 5 mM ATP treatment, a significant increase in the rate of DCF fluorescence was detected compared with vehicle control (Fig. 3A). To investigate whether ATP induced ROS generation is due to P2X7R activation, we treated naive CD4+ T lymphocytes with ATP in the presence of DPI (10 μM) or DCMO (10 μM) for 30 min. Treatment with ATP caused an increase in the rate of ROS generation compared with vehicle control (Fig. 3B). Treatment with DPI (10 μM) for 30 min significantly inhibited ATP-induced ROS generation (Fig. 3B). These results suggest that ATP-induced ROS generation is due to P2X7R activation.

To further investigate the role of ATP-induced ROS generation in CD62L shedding, we treated naive CD4+ T lymphocytes with ATP in the presence of DPI (10 μM) or DCMO (10 μM) for 30 min. Treatment with ATP caused an increase in the rate of ROS generation compared with vehicle control (Fig. 3C). Treatment with DPI (10 μM) for 30 min significantly inhibited ATP-induced ROS generation (Fig. 3C). These results suggest that ATP-induced ROS generation is due to P2X7R activation.
cene was observed \((n = 3; p < 0.05)\). DCF detects a variety of intracellular ROS species including \(H_2O_2\), hydroxyl radicals, peroxyl radicals, ONOO\(^-\), and NO. Previous studies have reported \(H_2O_2\) to cause CD62L downregulation \((42)\), and in naive CD4\(^+\) T lymphocytes, we observed this effect to be rapid upon stimulation with 100 \(\mu M\) \(H_2O_2\) (Fig. 3B).

We used a number of small molecule inhibitors of ROS generating enzymes to determine whether ROS generation through ATP stimulation caused CD62L downregulation. Unexpectedly, pretreatment with DPI, an inhibitor of flavone containing enzymes that include NADPH oxidase and complex I of the mitochondrial respiratory chain, caused a significant enhancement of ATP-induced CD62L downregulation (Fig. 3C). Remarkably, the NADPH oxidase inhibitor apocynin had no effect on ATP-induced CD62L loss, suggesting the enhancing DPI effect is independent of NADPH oxidase \((n = 3; p > 0.05)\) (Fig. 3D). Rotenone, an uncoupler of mitochondrial electron transport at complex I, also enhances ATP-induced CD62L downregulation in a concentration-dependent manner, suggesting DPI is acting through complex I (Fig. 3E). Interestingly, pretreatment with rottlerin, a nonspecific PKC inhibitor that also modulates ROS generation \((43)\), also caused significant increase in ATP-induced CD62L downregulation (Supplemental Fig. 2D). Antimycin A inhibits complex III, and pretreatment with this compound also led to a significant enhancement of ATP-induced CD62L loss (Fig. 3F). Importantly, DPI, rotenone, and antimycin A had no significant effect on basal CD62L surface expression, and the P2X7R inhibitor A438079 inhibited their enhancing effect on the ATP response (Fig. 4A, 4B). This suggests that these compounds require P2X7R activation to alter CD62L surface expression. ROS can affect the function of a number of ion channels including P2X2R, and we postulated that rotenone and antimycin A may be affecting P2X7R sensitivity \((44, 45)\). To confirm this, we treated naive CD4\(^+\) T lymphocytes with increasing concentrations of ATP following pretreatment with vehicle (DMSO), 5 \(\mu M\) rotenone, or 1 \(\mu M\) antimycin A and measured CD62L expression (Fig. 4C). We observed a leftward shift in the concentration response curves for ATP with rotenone and antimycin A compared with DMSO pretreatment. This indicates an increase in the potency of ATP in the presence of rotenone and antimycin A, as reflected by the lower \(EC_{50}\) for ATP in the presence of rotenone and antimycin A (both 0.43 mM) compared with DMSO (1.58 mM). This provides evidence that these compounds enhance ATP potency in the process of CD62L downregulation.

**Uncoupling of complex I and III from mitochondrial electron transport causes enhanced \(O_2^-\) generation**

Mitochondrial electron transport under normal physiological conditions causes the leakage of a small number of electrons which can react with \(O_2\) to form the ROS \(O_2^-\). In diseases driven by mutations to mitochondrial DNA or where increased ROS generation cause mitochondrial damage, this mitochondrial \(O_2^-\) generation can significantly increase. Uncoupling of mitochondrial electron transport at complex I and III has been shown to cause significantly enhanced \(O_2^-\) generation \((46, 47)\). This led us to investigate mitochondrial \(O_2^-\) generation as a possible mechanism of the enhancing effect of rotenone and antimycin A. \(O_2^-\) levels were measured using the dye MitoSOX Red, which is targeted to the mitochondria and fluoresces when oxidized by \(O_2^-\). Treatment with rotenone or antimycin A caused a concentration-dependent increase in mitochondrial \(O_2^-\) generation \((p < 0.001; \text{Fig. 4D, 4E})\).

**FIGURE 4.** Rotenone and antimycin A enhance ATP-induced CD62L downregulation and increase mitochondrial \(O_2^-\) production. Naive CD4\(^+\) T lymphocytes \((1 \times 10^6 \text{ cells/ml})\) were pretreated with DMSO or A438079 (10 \(\mu M\)) and antimycin A (1 \(\mu M\)) \((A)\) or rotenone (5 \(\mu M\)) \((B)\). Cells were then treated with ATP (3 mM) for 1 h, and CD62L surface expression was measured using flow cytometry. \((C)\) Cells were pretreated with DMSO, antimycin A (1 \(\mu M\)), or rotenone (5 \(\mu M\)) for 30 min prior to addition of ATP at the concentrations indicated for 1 h, after which CD62L expression was measured using flow cytometry. Cells were loaded with 2.5 \(\mu M\) MitoSOX Red, as described in Materials and Methods, and superoxide production was measured after 90 min treatment with rotenone \((D)\) or antimycin A \((E)\) at the concentrations indicated. Data are the mean of at least three independent experiments using cells from different donors ± SEM. One- or two-way ANOVA, followed by posthoc tests, was performed to compare significance differences between treatment groups. \(*p < 0.05\), \(*{*}p < 0.01\), \(*{*}*p < 0.001\), \(*{*}{*}p < 0.001\), \(*{*}{*}{*}p < 0.001\).

Rotenone and antimycin A reveal P2X7-dependent PS externalization

P2X7R activation in mouse CD4\(^+\) T lymphocytes has been linked to externalization of PS, which is normally confined to the inner leaflet of the plasma membrane. We did not observe significant PS “flopping” in response to 1-h 3 mM ATP treatment; however, pretreatment with 5 \(\mu M\) rotenone or 1 \(\mu M\) antimycin A, followed by ATP treatment, induced a significant P2X7R-dependent increase in PS surface exposure (Fig. 5). This indicates that the effect of rotenone and antimycin A on P2X7R function is not limited to CD62L downregulation. Previous studies with murine T lymphocytes have observed shrinkage of cells following ATP treatment \((48, 49)\); however, we did not observe this response in human naive CD4\(^+\) T lymphocytes (Supplemental Fig. 4B). In addition, treatment of cells with ATP did not lead to necrotic cell death (Supplemental Fig. 4C), measured by lactate dehydrogenase release, and pretreatment with rotenone did not enhance lactate dehydrogenase release from ATP-treated cells (Supplemental Fig. 4D). These data suggest that mitochondrial perturbation enhances the apoptotic marker PS but does not cause cells to undergo necrotic cell death.

![Image](http://www.jimmunol.org/DownloadedFrom/306x457-535x732)
Materials and Methods

PS exposure by Annexin V binding as described in prior to addition of ATP (3 mM) for 1 h. Cells were then analyzed for from different donors ± SEM. Two-way ANOVA, followed by posthoc test, to compare treatment groups. **

Discussion

In this study, we demonstrate the expression of P2X7R in human naive and activated CD4+ T lymphocytes at the protein level. This receptor is functional as demonstrated by concentration-dependent ATP-induced inward currents and loss of surface CD62L expression. The ATP responses occurred in response to concentrations in the low millimolar range that would be expected to activate P2X7R. Indeed, both ATP-stimulated inward current and loss of surface CD62L were sensitive to pretreatment with P2X7R antagonists. Remarkably, the loss of CD62L surface expression was insensitive to inhibitors targeting PKC, PI3K, MEK/ERK-1/2, molecules known to be involved in CD62L shedding induced by other agents (25–28). A broad-spectrum MMP antagonist inhibited loss of CD62L surface expression, suggesting that these proteases might be involved in shedding of this receptor in response to ATP/P2X7R stimulation. Some MMPs such as ADAM17 can be activated by ROS, which we demonstrated is elevated in naive CD4+ T cells following ATP treatment. Remarkably, use of small molecule inhibitors of mitochondrial electron transport chain such as rotenone and antimycin A led to a significant enhancement of mitochondrial O2− and oxidative stress, which correlated with enhanced sensitivity to ATP. Both rotenone and antimycin A enhanced ATP-induced CD62L downregulation. Cotreatment with rotenone or antimycin A with ATP also led to P2X7-dependent PS externalization, which did not occur with individual treatments.

P2X7R expressed on human naive CD4+ T lymphocytes acts as a functional ion channel, sensitive to divalent cations and inhibition by A438079. The inward current is initially rapid, followed by a second slower phase, when ATP is removed the current returns to resting levels after a short delay. This current profile is similar to that observed in Xenopus oocytes expressing P2X7R cloned from human B lymphocytes (50). Previous studies using voltage clamp electrophysiology investigated the divalent cation sensitivity of rat and human P2X7R expressed in HEK293 cells (3, 32). When cells were treated with 30 μM BzATP, Ca2+ and Mg2+ inhibited 50% of currents at 3.2 and 2.2 mM, respectively. A number of processes downstream of P2X7R activation by ATP are blocked by the presence of extracellular Mg2+ ions, including cell shrinkage and downregulation of cell surface CD62L and CD23 expression (19, 49, 51). Although removal of CaCl2 and MgCl2 from the extracellular solution potentiated P2X7R-mediated currents in this study, the absence of these divalent cations from the extracellular solution did not affect CD62L downregulation.

The Ab used to detect P2X7R expression in this study recognizes amino acid residues 331−595 of the C terminus of P2X7R. A recent study revealed that human P2X7R, like P2X4R, is alternatively spliced, and three of these eight isoforms have a truncated C-terminal domain (52, 53). Consequently, the anti-P2X7R Ab used in this study can potentially only recognize five of these isoforms, including the originally identified form, sometimes referred to as P2X7A receptor. PCR revealed that P2X7A is expressed in resting human CD4+ T lymphocytes along with P2X7B, which has a truncated C terminus (52). P2X7B has been cloned, and its function when expressed in HEK293 cells has been explored when expressed either alone or in combination with P2X7A (52, 53). Although P2X7B alone does not couple to pore formation or caspase activation, it can form heterotrimetric structures with P2X7A, which causes enhanced pore formation. In naive CD4+ T lymphocytes, we observe significant ethidium bromide incorporation (a measure of pore formation) in response to ATP, which is insensitive to inhibition by A438079 (Supplemental Fig. 4A). This may indicate that the pore formation in human naive CD4+ lymphocytes responding to ATP involves either splice variants of P2X7R and/or other purinergic receptors that are insensitive to A438079.

Several in vitro studies have investigated the potential source of endogenous extracellular ATP that may contribute to lymphocyte and immune cell activation in vivo (54, 55). Recent evidence suggests that following T lymphocyte activation, ATP is released through Pannexin-1 channels and acts in an autocrine manner to activate P2X receptors (13, 14). Indeed, a recent study showed that the bee venom component melittin causes release of ATP leading to P2X7R-dependent cleavage of E-cadherin and EGFR ligand from keratinocytes via ADAM10 and 17, respectively (56). In addition, cardiac fibroblasts subjected to hypotonic stress, release ATP through connexin channels, which in turn, acts on P2Y2 receptors in an autocrine manner to cause the release of profibrotic factors (57). The study of leukemic Jurkat T cells undergoing apoptosis revealed a novel mechanism of Pannexin-1 activation role of P2X7R and mitochondria in CD62L shedding

Role of P2X7R and Mitochondria in CD62L Shedding

FIGURE 5. Rotenone and antimycin A also enhance PS exposure in response to ATP. Naive CD4+ T lymphocytes (1 × 10^6 cells/ml) were pretreated with DMSO, rotenone (5 μM), or antimycin A (1 μM) for 30 min prior to addition of ATP (3 mM) for 1 h. Cells were then analyzed for PS exposure by Annexin V binding as described in Materials and Methods. Data are the mean of at least three independent experiments using cells from different donors ± SEM. Two-way ANOVA, followed by posthoc test, to compare treatment groups. *p < 0.01.

FIGURE 6. Effect of uncouplers of complexes I and III of the mitochondrial electron transport on ATP-induced loss of cell surface CD62L expression and PS exposure. (1) ATP induces loss of cell surface CD62L expression through P2X7R. (2) Rotenone and antimycin A are uncouplers of complexes I and III of the mitochondrial respectively. (3) Rotenone and Antimycin A significantly increase mitochondrial superoxide levels. (4) Pretreatment with these compounds or DPI, an inhibitor of flavin containing enzymes such as complex I, but not apocynin (5), an NADPH oxidase inhibitor, significantly enhance ATP-induced CD62L downregulation. (5) Rotenone or antimycin A reveals P2X7R-dependent externalization of PS.
that involves C-terminal cleavage of this hemichannel by caspases (58). This could represent a mechanism where ATP acts as a “find-me” signal to promote the clearance of apoptotic cells, through recruitment of phagocytes (59). ATP could potentially reach millimolar concentrations locally when released from Pannexin-1 channels in an autocrine or pancrine manner from healthy or apoptotic cells, respectively. In vitro techniques to measure concentrations of ATP release following stimulation have been developed (54), and techniques are now emerging to measure in vivo levels of external ATP (60). It will be critical to measure external ATP concentrations in SLOs and the periphery under normal and inflammatory conditions to further understand the contribution of lymphocyte P2X7R to inflammatory responses.

Pharmacological uncoupling of mitochondrial electron transport at complexes I and III leads to oxidative stress that enhances lymphocyte P2X7R downstream signaling events. This is consistent with previous reports that inhibition of complex I or III by rotenone and antimycin A/myxothiazol could enhance P2X2R activation and which have implicated ROS in ion channel modulation (44, 45). The impact on P2X7R responsiveness to ATP could occur at two levels. First, oxidative stress may lead to direct effects on P2X7 similar to that observed in P2X2R where ROS potentiate receptor activity through intracellular C terminal Cys430 residue (44). Indeed, P2X7R has a cysteine-rich C terminal domain that is potentially sensitive to modulation by oxidative stress that leads to the potentiation of receptor activation (61). Alternatively, the site of interaction could be downstream of P2X7R activation and involve intermediary proteins regulated by P2X7R activation and sensitive to ROS. One such protein could be the stress-activated MAPK p38, which is phosphorylated in response to ATP (62). Indeed, in human monocytes, LPS and H2O2 induce ADAM17 activation through the p38 MAPK signaling pathway (25, 63).

Uncoupling of mitochondrial electron transport at complexes I and III in the presence of millimolar ATP also leads to significant PS exposure. In this study, the role of P2X7R in these processes was confirmed using the next generation antagonists A438079 and AZI16645373, which show improved selectivity compared with previous P2X7R inhibitors (64). PS is normally confined to the inner leaflet of the plasma membrane, cells undergoing apoptosis externalize PS, which acts as cue for phagocytes to engulf and destroy apoptotic material (65, 66). Interestingly, neither ATP alone nor uncoupling of complex I or III in the absence of ATP led to PS externalization. This is in contrast to studies of murine CD4+ T lymphocytes, which show significant PS externalization in response to ATP alone (67, 68). The ability of mitochondrial O2− to modulate P2X7R function may represent a novel protective mechanism. Cells that are under physiological conditions lose CD62L expression but do not externalize PS in response to extracellular ATP and can presumably function as normal. We hypothesize that during T lymphocyte activation, ATP is released via Pannexin-1 channels to act in an autocrine or pancrine manner to facilitate CD62L downregulation and allow egress of cells from SLOs. In the inflamed periphery, ATP released from damaged cells could lead to downregulation of CD62L from naïve or memory CD4+ T lymphocytes and prevent their entry into the lymphatic system. In contrast, cells undergoing oxidative stress, which are also exposed to high levels of extracellular ATP, respond by externalization of PS that may act as a “find-me signal” for efficient removal from the body by phagocytosis. We propose a model where P2X7R activation, under conditions of oxidative stress, adds to the resolution of inflammation under physiological or pathological conditions. Indeed, P2X7R has been implicated in a number of inflammatory and autoimmune disorders including rheumatoid arthritis, multiple sclerosis, ALS, and systemic lupus erythematosus (69–73). Although these studies have been primarily in mouse models, where P2X7R may not be sensitive to modulation by ROS, evidence exists to suggest that P2X7R may also play a role in these diseases in humans (74). Interestingly, the pathobiology of some of these diseases involves a mitochondrial ROS component.

However, the contribution of lymphocyte P2X7R to inflammatory responses is complicated by the expression of single nucleotide polymorphisms (SNPs) that either increase or inactivate P2X7R function (75). The expression of P2X7R SNPs varies between individuals leading to differences in P2X7R function (76); indeed, expression of SNPs can determine the clearance of Mycobacterium tuberculosis by macrophages. In addition, the relative endogenous coexpression of full-length P2X7A compared with inactivating and enhancing P2X7R splice variants will influence ATP-mediated processes (52, 53). Indeed, we have observed that the magnitude of CD62L downregulation following ATP treatment can vary between healthy human donors (Supplemental Fig. 1E); this variation also extends to other processes such as ROS generation (data not shown). Inactive P2X7R would be unlikely to elicit this putative protective mechanism and as a consequence, cells damaged by excessive oxidative stress would avoid clearance and potentially enhance inflammation. It will be important to evaluate the contribution of P2X7R in conditions of oxidative stress to the overall inflammatory response particularly in inflammatory and autoimmune conditions associated with changes in mitochondrial function.

In summary, we present a novel mechanism involving modulation of P2X7R activity by mitochondrial oxidative stress that may be involved in the resolution of inflammation (Fig. 6). This protective mechanism should be considered in diseases where CD4+ T lymphocytes are exposed to increased ROS. In addition, the relative expression of P2X7R SNPs and splice variants in lymphocytes of inflamed patients may be of relevance in this model.

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I.K. is an employee of Pfizer Ltd. The other authors have no financial conflicts of interest.

References


Supplementary Figures

**Supplementary Figure 1.** Naïve CD4⁺ T lymphocytes (0.5x10⁶) were stained with IgG2a (allophycocyanin) and IgGκ1 (FITC) isotype control Abs or anti-CCR7 (allophycocyanin) and anti-CD62L (FITC) Abs. Staining for these Abs was assessed using flow cytometry and indicates cell surface expression of these molecules. A, Dot plot showing isotype control Abs and dual staining for CD62L and CCR7. B, Histogram of CD62L expression with IgGκ1 control. C, Histogram of CCR7 expression with IgG2a control. D, Naïve CD4⁺ T lymphocytes (1x10⁶/ml) were treated with PMA (100 nM) for 30 minutes or ATP (3 mM) for 1 hour, cell surface CD62L expression was measured by flow cytometry and is displayed using histograms. E. Naïve CD4⁺ T lymphocytes (1x10⁶/ml) were freshly isolated from two different donors on three separate occasions each. Cells were treated with ATP (3 mM) for 1 hour or anti-CD3/CD28 Ab coated beads (1 bead per cell) for 18 hours. Cell surface CD62L expression was then measured by flow cytometry. Data are the mean of 3 independent experiments using cells from two different donors ± SEM. One Way ANOVA, followed by Tukey’s post-hoc test was performed to compare significance differences between treatment groups in individual donors (ns) p>0.05 * p<0.05 **/## p<0.01 and Two Way ANOVA was used to compare donors ^ p<0.01.

**Supplementary Figure 2.** Naïve CD4⁺ T lymphocytes (1x10⁶/ml) were pre-treated for 30 minutes with DMSO or increasing concentrations of A, ZSTK474, B, PD98059, C, G66976 or D, Rottlerin, before 1 hour treatment with ATP (3 mM). Cell surface CD62L expression was analyzed by flow cytometry and. Data are the mean of at least 3 independent experiments using cells from different donors ± SEM. One Way ANOVA, followed by Tukey’s post-hoc test was performed to compare significance differences between treatment groups */# p<0.05.
Supplementary Figure 3. A, Naïve CD4+ T lymphocytes (1x10^6/ml) were treated with increasing concentrations of ATP for 1 hour in external buffer containing 1.2 mM CaCl₂ or nominally free of CaCl₂. Cell surface CD62L expression was measured using flow cytometry. Two Way ANOVA was used to compare treatment groups p>0.05. B, Naïve CD4+ T lymphocytes (1x10^6/ml) were treated with increasing concentrations of Thapsigargin for 1 hour. Data are the mean of at least 3 independent experiments using cells from different donors ± SEM. One Way ANOVA, followed by Tukey’s post-hoc test was performed to compare significance differences between treatment groups *** p<0.001.

Supplementary Figure 4. A. Naïve CD4+ T lymphocytes (1x10^6/ml) were re-suspended in external buffer solution (as described in Materials and Methods for electrophysiology) plus 25 μM ethidium bromide. Cells were then pre-treated with vehicle or A438079 (10 μM) for 30 minutes, followed by ATP (5 mM) for 15 minutes. Fluorescence was monitored using a multi-detection plate reader (Fluostar Optima, BMG Labtech, UK; excitation, 544 nm; emission, 590 nm). Addition of 0.2 % Triton X-100 was used to normalize fluorescence as a percentage of maximum ethidium bromide uptake. Measurements were performed in triplicate per treatment group. Linear regression was performed to determine the rate of ethidium bromide uptake. B, Cells were treated with vehicle or increasing concentrations of ATP for 1 hour, cells were then analyzed by flow cytometry and the cell size calculated by measuring forward scattered light. C, Cells were treated with vehicle increasing concentrations of ATP for 1 hour. D, Alternatively, cells were pre-treated with vehicle or Rotenone (5 μM) for 30 minutes followed by 3 mM ATP for 1 hour. In both cases necrotic cell death was measured by the levels of LDH released into the supernatant.
Supplementary Figure 1
Supplementary Figure 2
Supplementary Figure 4