“HOST TISSUE DAMAGE” SIGNAL ATP PROMOTES NON-DIRECTIONAL MIGRATION AND NEGATIVELY REGULATES TOLL-LIKE RECEPTOR SIGNALING IN HUMAN MONOCYTES
Andreas Kaufmann‡, Boris Musset§, Sven H. Limberg§, Vijay Renigunta§, Rainer Sus§, Alexander H. Dalpke¶, Klaus M. Heeg¶, Bernard Robaye** and Peter J. Hanley§
From the ‡Institute of Immunology, Marburg University, Robert-Koch-Str. 17, 35037 Marburg, Germany; §Institute of Physiology, Marburg University, Deutschhausstrasse 2, 35037 Marburg, Germany; ¶Hygiene-Institut, Im Neuenheimer Feld 324, 69120 Heidelberg, Germany; and **Institute of Interdisciplinary Research, Institut de Biologie et de Médecine Moléculaires, Université Libre de Bruxelles, Gosselies, Belgium
Running Title: Regulation of human monocytes by extracellular ATP
Address correspondence to: Peter J. Hanley, Institute of Physiology, Marburg University, Deutschhausstrasse 2, 35037 Marburg, Germany, Tel. +49-6421-286-6546; Fax. +49-6421-286-8960; E-Mail: hanley@mailer.uni-marburg.de

Activation of Toll-like receptors (TLRs) by lipopolysaccharide (LPS) or other ligands evokes a proinflammatory immune response which is not only capable of clearing the invading pathogens but can also inflict damage to host tissues. It is therefore important to prevent overshoot of the TLR-induced response where necessary, and here we show that extracellular ATP is capable of doing this in human monocytes. Using RT-PCR, we showed that monocytes express P2Y1, P2Y2, P2Y4, P2Y11 and P2Y13 receptors, as well as several P2X receptors. To elucidate the function of these receptors, we first studied Ca\(^{2+}\) signaling in single cells. ATP or UTP induced a biphasic increase in cytosolic Ca\(^{2+}\) which corresponded to internal Ca\(^{2+}\) release followed by activation of store-operated Ca\(^{2+}\) entry. The evoked Ca\(^{2+}\) signals stimulated Ca\(^{2+}\)-activated K\(^+\) channels, producing transient membrane hyperpolarization, and induced pseudopodia retraction. In addition, ATP promoted cell migration, however, unlike chemoattractants, the migration was non-directional and further analysis showed that ATP did not activate Akt, essential for sensing gradients. When TLR2, TLR4 or TLR2/6 were stimulated with their respective ligands, ATPγS profoundly inhibited secretion of proinflammatory cytokines (tumour necrosis factor-\(\alpha\) and monocyte chemoattractant protein-1), but increased production of interleukin-10, an anti-inflammatory cytokine. In radioimmune assays, we found that ATP (or ATPγS) strongly increased cAMP levels and, moreover, the TLR-response was inhibited by forskolin whereas UTP neither increased cAMP nor inhibited the TLR-response. Thus, our data suggest that ATP promotes non-directional migration and, importantly, acts as a “host tissue damage” signal via the Gs protein-coupled P2Y11 receptor and increased cAMP to regulate negatively TLR-signaling.

Innate immune cells express various surface membrane receptors which enable them to respond to changes in their environment, such as invasion by pathogens. Among these receptors are the evolutionarily conserved Toll-like receptors (TLRs), which recognize specific molecular patterns such as bacterial components. TLR4 recognizes lipopolysaccharide (LPS), a major structural component of the outer membrane of Gram-negative bacteria (1,2). Stimulation of TLR4 by LPS, also known as endotoxin, can initiate a proinflammatory immune response which serves to clear bacterial infection but if the response is not sufficiently tamed it can also inflict damage to host tissues, culminating in life-threatening endotoxic shock (3). Accordingly, endotoxic shock can be simulated by exposing mice to LPS whereas TLR4-/- (TLR4-deficient) mice are hyporesponsive to this ligand (4). TLR4-signaling is initiated when a complex of LPS and LPS-binding protein binds to the receptor CD14, which is strongly expressed in monocytes, and also present in macrophages and dendritic cells. Alternatively, soluble CD14, a serum protein, can substitute for membrane-bound CD14 and initiate TLR4-signaling in cells lacking CD14, such as endothelial cells. The secreted protein MD-2, which binds to the extracellular domain of TLR4 is also essential for responsiveness to LPS, which is virtually absent in MD-2-/- mice (5,6). Although the molecular mechanisms by which LPS and other ligands stimulate TLRs leading to a specific pattern of gene expression are well understood (7), detailed information about its
negative regulation is lacking. Stimulation of TLR4, as well as other TLRs, activates IRAKs (IL-1 receptor-associated kinases) via the adaptor protein MyD88 (myeloid differentiation primary-response protein 88) which leads to downstream activation of the IKK complex (inhibitor of IkB kinase complex), which consists of IKK-α, IKK-β and IKK-γ. The upstream signaling has been confirmed with MyD88-/- or IRAK4-/- mice which show little response to LPS (8,9). The IKK complex phosphorylates the inhibitory protein IkB (inhibitor of (nuclear factor) κB), which leads to its subsequent degradation, allowing release of NF-κB (nuclear factor κB), which translocates to the nucleus and promotes the expression of inflammatory cytokines. Following stimulation of TLRs in monocytes or macrophages, expression of IRAK-M increases which has been shown to regulate negatively TLR-signaling, as have upregulation of SIGIRR (single immunoglobulin IL-1 receptor-related molecule) (10) and SOCS1 (suppressor of cytokine signaling-1) (11,12). The latter, though, probably only dampens secondary (interferon-β cytokine signaling-1) (11,12). The latter, though, probably only dampens secondary (interferon-β-dependent) pathways (13,14). In any case, these negative regulators do not convey information about the state of host tissues.

Recent studies with human monocyte-derived dendritic cells (DCs) suggest that extracellular ATP is capable of negatively regulating TLR-signaling via extracellular nucleotide receptors, which can be broadly divided into the P2Y and P2X receptor families. In DCs, ATP has been shown to inhibit LPS-induced secretion of the proinflammatory cytokines TNFα and IL-12 (15-17), which raises the possibility that ATP may act, among other roles, as a “host tissue damage” signal for innate immune cells and inhibit TLR-signaling in a negative feedback fashion. To test this possibility and to understand better the effect of extracellular ATP on innate immunity, we have investigated the effects of nucleotide receptor activation on the migration, second messenger signaling and cytokine secretion of human monocytes in the absence and presence of various TLR ligands.

Materials and Methods

Isolation of Monocytes - Methods were performed essentially as recently described (18). In brief, human peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy volunteers by density centrifugation (Ficoll-Hypaque, 1.077 g/ml, Biochrom). Monocytes were further enriched to a purity of ~95 % by counterflow centrifugation (19). Cells were plated in cell culture dishes containing Clinks/RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10 % heat-inactivated FCS (Biochrom, Berlin). Monocytes of maximal purity (> 95 %) were isolated using anti-CD14 MicroBeads (Miltenyi Biotec, Germany) in a SuperMACS.

Analysis of Gene Expression - Total cellular RNA was prepared from CD14+ monocytes using a HighPure RNA extraction kit (Roche, Mannheim, Germany) which included Dnase I digestion. After reverse transcription with a cDNA synthesis kit (MBI Fermentas St. Leon-Rot, Germany), PCR (polymerase chain reaction) analysis was performed using primers specific for P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12; and P2Y13; and P2X1, P2X3, P2X5, P2X4, P2X6 and P2X7. The PCR primers have recently been published (18), except for P2Y13 which was: TTGTTTTTCATCTCCCTGCAAAATA. Real-time quantitative PCR was done using a Eurogentec qPCR kit and measured values were normalized to the level of GAPDH (glyceraldehyde 3-phosphate dehydrogenase) RNA expression.

Single-Cell Ca2+ Imaging - A cover slip seeded with monocytes was sealed onto the bottom of a Perspex bath (volume, 100 µl) mounted on the stage of an inverted microscope (Nikon Diaphot 300). Cells were imaged via a x40 (1.4 numerical aperture) oil-immersion Nikon objective lens and superfused at 1 ml/min with solution containing 5 % BSA (bovine serum albumin) and (mM): 140 NaCl, 5.4 KCl, 1 MgCl2, 0.33 NaH2PO4, 5 HEPES, 1 CaCl2, 0.5 probenecid and 10 glucose (pH, 7.4). After 15 min incubation with 10 µM fluo-3/AM (Molecular Probes), a single monocyte was selected and excited at 488 nm via a monochromator while fluorescence was detected at 530 ± 15 nm. Only one cell per cover slip was used for experiments. The fluorescence signals were normalized with respect to the resting fluorescence intensity (F0) and expressed as F/F0.

Patch-Clamp Measurements - Monocytes were superfused with solution containing (mM): 140 NaCl, 4.5 KCl, 1.2 NaH2PO4, 1.13 MgCl2, 1.6 CaCl2, 10 HEPES, 10 glucose, 2 Na-pyruvate and 0.5 % BSA (pH, 7.4) or with high-K+ solution containing 140 mM KCl and 4.5 mM NaCl. The pipette solution contained (mM) 50 KCl, 65 K+ glutamate, 10 KH2PO4, 2 MgCl2, 1.9 K3ATP, 0.2 Na3-GTP, 0.1 EDTA and 5 HEPES (pH, 7.2). Data were acquired using an Axopatch.
% CO₂, cells were fixed with methanol and incubation at 37 ºC in air containing phenylalanine) was used as a positive control. NFMLP (formyl-methionyl-leucyl-phenylalanine) was used as a positive control. After 1 h incubation at 37 ºC in air containing 5% CO₂, cells were fixed with methanol and stained with hematoxylin (Sigma). A software-controlled imaging system (Vilber Lourmat; distributed by Fröbel, Wasserburg, Germany) was used to quantify the number of migrated monocytes per well. Results are expressed as the number of stimuli-induced migrated cells after subtracting unspecific migration (negative control).

Time-Lapse Microscopy - Images were acquired using a combination of differential interference contrast (DIC) and time-lapse microscopy using an Olympus IX71 microscope equipped with a software-controlled (Image-Pro Plus 4.5, Media Cybernetics, Silver Spring, MD, USA) SensiCamQE CCD camera (Chromaphor, Duisburg, Germany). Monocytes were plated on collagen type I-coated coverslips and imaged using a x60 (1.4 numerical aperture) Olympus objective lens and immersion oil.

Western Blot Analysis - Monocytes (5 x 10⁶/well) were incubated for 20 h in 6-well tissue culture plates and then either left untreated or stimulated with 100 µM ATP, 100 µM ATP₇S or 0.1 µM FMLP. After 3 min, cells were lysed using a protein extraction reagent (M-PER, Pierce Biotechnology, Rockford, IL.), supplemented with a protease inhibitor mixture (Complete, Roche Diagnostics, Mannheim, Germany). Phosphorylation of Akt was then assayed by Western blot as recently described (21). Equal loading was controlled by detecting total Akt.

cAMP Measurements - Monocytes were preincubated for 30 min in RPMI-1640/HEPES buffer containing 25 µM rolipram for 30 min and then treated with a nucleotide or forskolin in the same medium for 15 min. Activity was stopped by adding 0.1 M HCl and cAMP was quantified by radioimmune assays after acetylation.

Cytokine Measurements - Secretion of cytokines into the culture supernatants was determined by sandwich ELISA (enzyme-linked immunosorbent assay), as described previously (19,20). In brief, 96-well microtiter plates (Maxisorp, Nunc, Wiesbaden, Germany) were coated with cytokine-specific monoclonal antibodies specific for TNFα, MCP-1 or IL-10 (PharMingen, Hamburg, Germany). Plates were blocked with 2% BSA in PBS. Aliquots of culture supernatants (100 µl/well) were incubated at room temperature for 1 h, and then a specific biotinylated secondary antibody (PharMingen) and a streptavidin-peroxidase complex was added. Conversion of the substrate ortho-phenylenediamine dihydrochloride was measured using a Dynatech MR7000 microplate reader. Sensitivities of the ELISAs were: <3 pg/ml (IL-10), <20 pg/ml (MCP-1) and <100 pg/ml (TNFα).

Infection with influenza A virus – Influenza A/PR/8 (H1N1) virus was propagated and purified as previously described (22). Viral titers were determined by plaque assay and monocytes were infected with H1N1 virus at a multiplicity of infection of 2.

Cell Viability Assay – Cell viability was assessed using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After 16 h of incubation under control or treatment conditions, cells were washed twice with PBS and further incubated for 2 h with 0.5 mg/ml MTT. After cell lysis and solubility of crystals with isopropanol, reduction of MTT was determined by measuring absorption at 570 nm using a microplate reader. Cell viability, assessed by MTT reduction, was expressed as % control.

RESULTS

At least seven human P2Y receptors have been identified and characterized: P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂ and P2Y₁₃. We found that monocytes express P2Y₂ and P2Y₁₁, similar to macrophages (18), as well as the recently described Gi-coupled P2Y₁₃ receptor (23) (Fig. 1A). Weak signals for P2Y₁ and P2Y₄ were also detected. Furthermore, monocytes were found to express the ligand-gated ion channels P2X₁, P2X₄ and P2X₇ (Fig. 1B). Most of the P2Y receptor subtypes are coupled to PLC and Ca²⁺.
signaling, however, the P2Y₁₁ receptor is additionally coupled to adenylate cyclase by the stimulatory G protein Gs (24) whereas the P2Y₁₃ receptor is negatively coupled to this enzyme (23,25). Since extracellular ATP can be hydrolyzed to ADP by CD39 (ecto-nucleoside triphosphate diphosphohydrolase-1), the major ectonucleotidase of monocytes, we tested whether or not ATP (10 and 100 µM) caused a net increase in cAMP levels. Indeed, ATP and its non-hydrolyzable analogue ATPγS both strongly increased cAMP in monocytes (Fig. 1C) suggesting that the P2Y₁₁/Gs/adenylate cyclase pathway was functionally dominant in these cells.

In whole-cell current-clamp recordings, application of 10 µM ATP consistently induced a transient depolarization (n = 13) which was followed by hyperpolarization (Fig. 1D). The initial depolarization was probably due to activation of P2X receptors because in 5 out of 7 cells it was absent when UTP was substituted for ATP (Fig. 1E). The secondary hyperpolarization was almost certainly due to the opening of Ca²⁺-activated K⁺ (KᵥCa) channels, which we have recently characterized in detail (18). Indeed, we found that monocytes express SK-4 (KCNN4) and BKᵥCa (KCNMA1) channels (Fig. 1F), similar to macrophages.

We have also recently shown that ATP induces Ca²⁺ oscillations in human macrophages which may regulate various enzymes and gene expression (18). Surprisingly, application of ATP to single human monocytes did not induce such an oscillatory release of Ca²⁺, but typically we observed a rapid Ca²⁺ transient consisting of 1-3 spikes followed by a slower secondary increase (Fig. 2A). The slower secondary increase, also observed with UTP (inset, Fig. 2A), was probably due to store-operated Ca²⁺ entry since it was absent in Ca²⁺-free solution and it could be seen when Ca²⁺ was subsequently introduced (Fig. 2B). Ca²⁺ influx consistent with store-operated Ca²⁺ entry could also be repeatedly observed when the sarco(endo)plasmic reticular Ca²⁺-ATPase was blocked with thapsigargin (500 nM) and Ca²⁺ was intermittently introduced (see inset, Fig. 2B). Figures 2C and D show that ATP and UTP equipotently increase Ca²⁺ with half-maximal response at ~0.1 µM. Using digital time-lapse microscopy, we observed that the application of either ATP (n = 10) or UTP (n = 4) caused strong retraction of pseudopodia (single frames shown in Fig. 3A and B; see also time-lapse movie in Supplemental Data), even in the absence of external Ca²⁺. This observation suggested that ATP may act as a chemoattractant.

We found in chemotaxis assays that ATP promoted cell migration in the concentration range 10-100 µM, whereas higher concentrations were inhibitory (Fig. 3C). In parallel, we performed checkerboard analyses (not shown) which revealed that ATP did not induce directional migration. That is, the stimulatory effect of ATP on migration was still observed when it was added at equal concentrations to both sides of the chamber. Although both P2Y and FMLP receptors are coupled by Gq to PLC (phospholipase C), which hydrolyzes phosphatidylinositol 4,5-bisphosphate, yielding diacylglycerol and inositol 1,4,5-trisphosphate, this signaling pathway alone is not sufficient to induce migration along chemotactic gradients. The FMLP receptor is additionally coupled to PI3K (phosphoinositide 3-kinase), which through the generation of PIP₃ (phosphatidylinositol 3,4,5-trisphosphate) activates Akt (also known as protein kinase B), the activity of which is necessary for cell navigation (26-29). For example, in studies using PI3Kγ−/− mice, directional movement of neutrophils in FMLP gradients is severely impaired without affecting random movement (29). To test the hypothesis that ATP differs from FMLP in that it does not activate the PI3K-Akt pathway we performed immunoblot analysis. Stimulation of monocytes with either ATP or ATPγS did not produce phosphorylation of Akt whereas FMLP produced a strong “positive control” response (Fig. 3D).

We next investigated the effects of extracellular nucleotides on TLR-signaling. Monocytes were stimulated with 10 ng/ml LPS in the presence or absence of various concentrations of ATPγS or UTP for 16 h. ATPγS or UTP alone had no significant effect on TNFα production (Fig. 4A). However, ATPγS, but not UTP, inhibited LPS-induced production of TNFα when it was added at equal concentrations to both sides of the chamber. Although both P2Y and P2Y₄ receptors, where UTP ≥ ATP are agonists (30), are involved. The P2Y₁ and P2Y₁₃ receptors are also unlikely to be involved since both of these receptors are activated preferentially by diphosphate adenine nucleotides. Moreover, the P2Y₁ blocker MRS2179 (100 µM) did not reduce the ability of ATPγS to inhibit LPS-induced production of TNFα (Fig. 4B). It could be argued that hydrolysis-resistant ATPγS is slowly degraded to adenosine, which could confound the interpretation of data. However, the activity
of CD73 (ecto-5′-nucleotidase), which catalyzes the hydrolysis of AMP to adenosine, is weak in monocytes (31) and, moreover, we found that TLR-signaling was similarly inhibited when experiments were repeated in the presence of 100 μM 8-p-SPT (8-(p-sulfophenyl)theophylline), an adenosine receptor blocker (not shown). Thus, P2Y11 is most likely the receptor through which ATP inhibits TLR-signaling. Consistent with this conclusion, the potent P2Y11 receptor agonist BzATP (3′-O-(4-benzooylbenzoyl) ATP), as well as the weaker agonist ADPβS (32), inhibited TLR-signaling (Fig. 4B).

Because the P2Y11 receptor is coupled to adenylate cyclase, we speculated that an increase in cAMP may inhibit the TLR-signaling pathway. In support of this notion, direct activation of adenylate cyclase with 100 μM forskolin inhibited LPS-induced TNFα production, but less effectively than ATPγS (Fig. 4C). When cells were pretreated with the PKA blocker H89 (10 μM), the inhibitory effect of ATPγS on LPS-induced TNFα production was still observed (Fig. 4D). Hence, based on this observation, cAMP probably inhibits the activation and/or action of NF-κB independent of PKA. To test whether ATP inhibits LPS-induced TNFα production at the pretranslational level we performed quantitative real-time PCR. Figure 4E shows that LPS-induced expression of TNFα mRNA was significantly inhibited by ATPγS. ATPγS, but not UTP, also inhibited LPS-induced production of MCP-1 (Fig. 5A), which could be mimicked with forskolin. As was the case for TNFα, the inhibition of LPS-induced MCP-1 production by ATPγS was not blocked by either 8-p-SPT or H89 (Fig 5B). Furthermore, BzATP was equally effective as ATPγS (Fig 5B). Interestingly, ATPγS and ADPβS did not inhibit LPS-produced IL-10, but rather, the agonists synergized with LPS to increase secretion of this anti-inflammatory cytokine (Fig. 5C and D). This synergism was not blocked by either MRS2179 or 8-p-SPT (Fig. 5D).

Together, the above studies with the ligand LPS suggest that P2Y receptor agonists such as ATPγS inhibit TLR4-signaling. We extended these studies and tested whether ATPγS can inhibit the proinflammatory response mediated by other TLR receptors. Indeed, the production of TNFα by monocytes stimulated with 100 pg/ml macrophage-activating lipopeptide-2 (MALP-2), a TLR2/6 ligand (1), or 1000 ng/ml lipoteichoic acid (LTA), a TLR2 ligand (33), was inhibited by ATPγS (Fig. 6A and B). MCP-1 production induced by either MALP-2 (Fig. 6C) or LTA (Fig. 6D) was also inhibited by ATPγS. As was the case with LPS, ATPγS synergized with MALP-2 (Fig. 6E) or LTA (Fig. 6F) to increase IL-10 production.

The negative effect of ATPγS on inflammatory cytokine production induced by stimulating TLR2, TLR4 or TLR2/6 cannot be explained by global depression of cell function since IL-10 was increased in all cases. Moreover, ATPγS did not inhibit, but rather potentiated, MCP-1 production in monocytes stimulated by 10 U interferon-γ (IFN-γ), which is not a TLR ligand (Fig. 7A). Similarly, ATPγS did not inhibit the MCP-1 response of cells infected with influenza A (H1N1) virus (Fig. 7B), which incidentally, as expected, decreased cell viability (Fig. 7C). Note that viral nucleic acids may stimulate TLR3 (double-stranded RNA) or TLR8 (single-stranded RNA), receptors which are not located on the cell surface (7). Indeed, Guillot et al. (34) have recently shown that TLR3 is involved in the cellular response to influenza A (H3N2) virus.

At high concentrations (> 0.5 mM) ATP (or ATPγS) can induce cell apoptosis via P2X7 receptor activation (35,36). However, at the concentrations used in this study (≤ 0.1 mM), ATPγS did not affect cell viability (Fig. 7D). The apparent positive effect of LPS on cell viability (Fig. 7D) assessed by MTT reduction (an index of mitochondrial activity) suggests that TLR-signaling and increased cytokine production stimulate energy metabolism.

DISCUSSION

We have shown that ATP promotes “non-directional” migration and acts as an extracellular regulator of TLR-signaling in peripheral human monocytes. In chemotaxis assays we found that FMLP and ATP promoted migration, although checkerboard analyses revealed that monocytes could not sense ATP concentration gradients. The surface receptors for FMLP and ATP are each coupled to PLC, but recent studies with knockout mice have suggested that the PLC pathway is not sufficient for chemotaxis (27), the recognition and response to chemoattractant gradients. Instead, work with neutrophils and peritoneal macrophages isolated from PI3K-/- mice have indicated that PI3K is essential for chemotaxis (26-28), probably by promoting PIP3 accumulation and localization of Akt at a new leading edge (37). Consistent with this picture,
we found by Western blot analysis that FMLP, but neither ATP nor ATPγS, increased Akt phosphorylation. We also observed by means of time-lapse DIC microscopy that monocytes, plated on a collagen matrix, retracted their pseudopodia when ATP was applied, even in Ca²⁺-free solution. Hence, stimulation of the PLC pathway and IP₃-induced Ca²⁺ release probably promotes release of contacts and rapid actin reorganization, a prerequisite for migration. We also found that the pattern of Ca²⁺ signaling induced by ATP in human monocytes was distinctly different to that recently observed in human monocyte-derived macrophages (18). In contrast to macrophages, ATP did not evoke sustained Ca²⁺ oscillations in monocytes. Furthermore, the initial Ca²⁺ transient, which was independent of external [Ca²⁺]ᵢ, was followed by a second slower Ca²⁺ increase which decayed slowly. This slower secondary Ca²⁺ transient was abolished in Ca²⁺-free solution but it could be evoked by reintroduction of Ca²⁺, suggesting that it reflected the opening of Ca²⁺ release-activated Ca²⁺ channels rather than P2X channel activity. Although we detected expression of several P2X receptors, our patch-clamp recordings showed only a transient depolarization when ATP was applied. Since P2X₁ desensitizes rapidly within 1-2 s (38), and P2X₇ receptors are half-maximally activated by > 300 μM ATP in the presence of physiological Ca²⁺ and Mg²⁺ concentrations (39,40), we infer that P2X₄ receptors were mainly responsible for this transient depolarization. In addition to promoting migration, extracellular ATP potently inhibits the TLR-induced proinflammatory immune response of monocytes. Low micromolar concentrations of ATPγS markedly decreased LPS-induced production of TNFα and MCP-1, both of which are proinflammatory, whereas IL-10 secretion was augmented. In accord, several recent studies with DCs have shown that ATP can inhibit LPS-induced secretion of inflammatory cytokines (15-17). Aside from negatively regulating TLR4-signaling, we found using other TLR ligands that ATPγS also inhibited TLR2- and TLR6-signaling. However, ATPγS did not impair the cytokine response to viral infection, the nucleic acids of which can activate TLR3 and TLR8. In contrast to the TLR3-signaling pathway (34), TLR2, TLR4 and TLR6 use the adaptor molecule MyD88 to activate an IRAK-dependent pathway, leading to release of NF-κB and increased production of inflammatory cytokines (7). At present, we do not know the exact mechanism by which nucleotides inhibit this inflammatory signal pathway. On one hand, we found that UTP had no effect on TLR-signaling, implying that P2Y₂ and P2Y₄ receptors, and the PLC pathway, are probably not important for the negative regulation. On the other hand, the adenylate cyclase activator forskolin mimicked the inhibitory action of ATP on the LPS-induced proinflammatory response suggesting that cAMP may be a key regulator of TLR-signaling. Consistent with this notion, we found that ATP and ATPγS increased cAMP levels, presumably via the activation the unique P2Y₁₁ receptor, unique in the sense that it is posi- tively coupled to both adenylate cyclase and PLC. In concordance, activation of β-receptors, (which are also coupled to adenylate cyclase by Gs) and caffeine (a phosphodiesterase inhibitor) have been shown to increase cAMP concentrations and to inhibit LPS-induced TNFα production by innate immune cells (41-43). Moreover, selective activation of adenosine A₂A receptors increases cAMP and exerts an anti-inflammatory action in various immune cells (44,45). In human THP-1 monocytes, application of the membrane permeable analogue dibutyryl-cAMP has been reported to inhibit LPS-induced expression of TNFα mRNA, without affecting translocation of NF-κB to the nucleus. Rather, further work with human umbilical vein endothelial cells, suggested that increased cAMP induced by forskolin inhibited NF-κB-mediated transcriptional activity (46). Hence, cAMP may target the ultimate step of the MyD88-dependent TLR-signaling cascade. We cannot rule out a role for P2X receptor activation in modulating TLR-signaling. Recent data obtained with murine RAW 264.7 macrophages suggest that P2X7 receptor activation may in fact enhance LPS-induced inflammatory cytokine production. Hu et al. (35) demonstrated that LPS-induced stimulation of NF-κB activity was reduced in cells pretreated with the P2X7 antagonist periodate-oxidized ATP. Moreover, NF-κB activity was increased more when macrophages were treated with both LPS and a P2X7 receptor agonist (BzATP) than with LPS alone (47). Hence, both P2Y and P2X receptors may regulate the innate immune response to LPS (48). One possibility is that ATP inhibits TLR-signaling at low concentrations (< 100 μM) via P2Y receptors, whereas at much higher concentrations it activates P2X7 receptors to boost inflammatory cytokine production. However, the regulation of innate immune...
responses by nucleotides may differ considerably between humans and mice since, for example, the mouse genome lacks P2Y11 receptors and there is no known equivalent Gs-coupled P2Y receptor in this species.

In conclusion, our data suggest a model (shown in Fig. 8) whereby ATP acts as a “host tissue damage” signal for innate immune cells. That is, when the TLR-induced proinflammatory response to bacterial infection is excessively strong that it damages host tissues, ATP is released into the extracellular space and negatively regulates the response by binding to P2Y11 receptors and increasing cytosolic cAMP, which either directly, or indirectly via cAMP-dependent PKA or possibly Epac (exchange protein directly activated by cAMP) (49), inhibits the production of inflammatory cytokines and concomitantly increases the expression of the anti-inflammatory cytokine IL-10. We cannot rule out a role for P2X receptors in this scheme but at this stage there is no obvious mechanistic link. Independent of TLR-signaling, ATP can act via P2Y receptors, which are coupled to PLC but not PI3K, to promote non-directional migration.
REFERENCES

1. Akira, S., Takeda, K. & Kaisho, T. (2001) Nat. Immunol. 2, 675-680.
2. Beutler, B. & Rietschel, E. T. (2003) Nat. Rev. Immunol. 3, 169-176.
3. van der Poll, T. & Lowry, S. F. (1995) Shock 3, 1-12.
4. Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. & Akira, S. (1999) J. Immunol. 162, 3749-3752.
5. Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K. & Kimoto, M. (1999) J. Exp. Med. 189, 1777-1782.
6. Nagai, Y., Akashi, S., Nagafuku, M., Ogata, M., Iwakura, Y., Akira, S., Kitamura, T., Kosugi, A., Kimoto, M. & Miyake, K. (2002) J. Exp. Med. 189, 1777-1782.
7. Akira, S. & Takeda, K. (2004) Nat. Rev. Immunol. 4, 499-511.
8. Kawai, T., Sato, S., Ishii, K. J., Coban, C., Hemmi, H., Yamamoto, M., Terai, K., Matsuda, M., Inoue, J., Uematsu, S., Takeuchi, O. & Akira, S. (2004) Nat. Immunol. 5, 1061-1068.
9. Suzuki, N., Suzuki, S., Duncan, G. S., Millar, D. G., Wada, T., Mirtsos, C., Takada, H., Wakeham, A., Itie, A., Li, S., Penninger, J. M., Wesche, H., Ohashi, P. S., Mak, T. W. & Yeh, W. C. (2002) Nature 416, 750-756.
10. Wald, D., Qin, J., Zhao, Z., Qian, Y., Naramura, M., Tian, L., Towne, J., Sims, J. E., Stark, G. R. & Li, X. (2003) Nat. Immunol. 4, 920-927.
11. Kinjo, I., Hanada, T., Inagaki-Ohara, K., Mori, H., Aki, D., Ohishi, M., Yoshida, H., Kubo, M. & Yoshimura, A. (2002) Immunity 17, 583-591.
12. Nakagawa, K., Naka, K., Tsutsui, H., Fujimoto, M., Kimura, A., Abe, T., Seki, E., Sato, S., Takeuchi, O., Takeda, K., Akira, S., Yamanishi, K., Kawase, I., Nakanishi, K. & Kishimoto, T. (2002) Immunity 17, 677-687.
13. Baetz, A., Frey, M., Heeg, K. & Dalpke, A. H. (2004) J. Biol. Chem. 279, 54708-54715.
14. Gingras, S., Parganas, E., de Pauw, A., Ihle, J. N. & Murray, P. J. (2004) J. Biol. Chem. 279, 54702-54707.
15. La Sala, A., Ferrari, D., Corinti, S., Cavani, A., Di Virgilio, F. & Girolomoni, G. (2001) J. Immunol. 166, 1611-1617.
16. Wilkin, F., Stordeur, P., Goldman, M., Boeynaems, J.-M. & Robaye, B. (2002) Eur. J. Immunol. 32, 2409-2417.
17. Marteau, F., Communi, D., Boeynaems, J.-M. & Suarez-Gonzalez, N. (2004) J. Leuk. Biol. 76, 796-803.
18. Hanley, P. J., Musset, B., Renigunta, V., Limberg, S. H.; Dalpke, A. H.; Sus, R., Heeg, K. M., Preisig-Müller, R. & Daut, J. (2004) Proc. Natl. Acad. Sci. USA 101, 9479-9484.
19. Sprenger, H., Meyer, R. G., Kaufmann, A., Bußfeld, D., Rischkowsky, E. & Gemsa, D. (1996) J. Exp. Med. 184, 1191-1196.
20. Kaufmann, A., Mühlradt, P. F., Gemsa, D. & Sprenger, H. (1999) Infect. Immun. 67, 6303-6308.
21. Salentin, R., Gemsa, D., Sprenger, H. & Kaufmann, A. (2003) J. Leukoc. Biol. 74, 252-259.
22. Nain, M., Hinder, F., Gong, J. H., Schmidt, A., Bender, A., Sprenger, H. & Gemsa, D. (1990) J. Immunol. 145, 1921-1928.
23. Marteau, F., Le Poul, E., Communi, D., Communi, D., Labouret, C., Savi, P., Boeynaems, J.-M. & Gonzalez, S. (2003) Mol. Pharmacol. 64, 104-112.
24. Communi, D., Govaerts, C, Parmentier, M. & Boeynaems, J.-M. (1997) J. Biol. Chem. 272, 31969-31973.
25. Communi, D., Gonzalez, N. S., Detheux, M., Brézillon, S., Lannoyn, V., Parmentier, M. & Boeynaems, J. M. (2001) J. Biol. Chem. 276, 41479-41485.
26. Hannigan, M., Zhan, L., Li, Z., Ai, Y., Wu, D. & Huang, C.-K. (2002) Proc. Natl. Acad. Sci. USA 99, 3603-3608.
27. Hirsch, E., Katanaev, V. L., Garlanda, C., Azzolino, O., Pirola, L., Silengo, L., Sozzani, S., Mantovani, A., Altruda, F. & Wymann, M. P. (2000) Science 287, 1049-1053.
28. Li, Z., Jiang, H., Xie, W., Zhang, Z., Smrcka, A. V. & Wu, D. (2000) Science 287, 1046-1049.
29. Sasaki, T., Irie-Sasaki, J., Jones, R. G., Oliveira-dos-Santos, A. J., Stanford, W. L., Bolon, B., Wakeham, A., Itie, A., Bouchard, D., Koziarek-Szust, I., Joza, N., Mak, T. W., Ohashi, P. S., Suzuki, A., Penninger, J. M. (2000) Science **287**, 1040-1046.
30. Ralevic, V. & Burnstock, G. (1998) Pharmacol. Rev. **50**, 413-492.
31. Sunderman, F. W. (1990) **Ann. Clin. Lab. Sci.** **20**, 123-139.
32. Communi, D., Robaye, B. & Boeynaems, J.-M. (1999) **Br. J. Pharmacol.** **128**, 1199-1206.
33. Schwandner, R., Dziarski, R., Wesche, H., Rothe, M. & Kirschning, C. J. (1999) **J. Biol. Chem.** **274**, 17406-17409.
34. Guillot, L., Le Goffic, R., Bloch, S., Escriva, S., Chignard, M. & Si-Tahar, M. (2005) **J. Biol. Chem.** **280**, 5571-5580.
35. Hu, Y., Fisette, P. L., Denlinger, L. C., Guadarrama, A. G., Sommer, J. A., Proctor, R. A. & Bertics, P. J. (1998) **J. Biol. Chem.** **273**, 27170-27175.
36. Bulanova, E., Budagian, V., Orinska, Z., Hein, M., Petersen, F., Thon, L., Adam, D. & Bulfone-Paus, S. (2005) **J. Immunol.** **174**, 3880-3890.
37. Merlot, S. & Firtel, R. A. (2000) **J. Cell. Sci.** **116**, 3471-3478.
38. North, R. A. (2002) **Physiol. Rev.** **82**, 1013-1067.
39. Chessell, I. P., Michel, A. D. & Humphrey, P. P. (1998) **J. Pharmacol.** **124**, 1314-1320.
40. North, R. A. & Suprenant, A. (2000) **Annu. Rev. Pharmacol.** **40**, 563-580.
41. Severn, A., Rapson, N. T., Hunter, C. A. & Liew, F. Y. (1992) **J. Immunol.** **148**, 3441-3445.
42. Elenkov, I. J., Wilder, R. L., Chrousos, G. P. & Vizi, E. S. (2000) **Pharmacol. Rev.** **52**, 595-638.
43. Horrigan, L. A., Kelly, J. P. & Connor, T. J. (2004) **Int. Immunopharmacol.** **4**, 1409-1417.
44. Khoa, N. D., Montesinos, M. C., Reiss, A. B., Delano, D., Awadallah, N. & Cronstein, B. N. (2001) **J. Immunol.** **167**, 4026-4032.
45. Sitkovsky, M. V., Lukashev, D., Apasov, S., Kojima, H., Koshiba, M., Caldwell, C., Ohta, A. & Thiel, M. (2004) **Annu. Rev. Immunol.** **22**, 657-682.
46. Ollivier, V., Parry, G. C. N., Cobb, R. R., de Prost, D. & Mackman, N. (1996) **J. Biol. Chem.** **271**, 20828-20835.
47. Aga, M., Watters, J. J., Pfeiffer, Z. A., Wiepz, G. J., Sommer, J. A. & Bertics, P. J. (2004) **Am. J. Physiol.** **286**, C923-C930.
48. Guerra, A. N., Fisette, P. L., Pfeiffer, Z. A., Quincha-Rios, B. H., Prabhu, U., Aga, M., Denlinger, L. C., Guadarrama, A. G., Abozeid, S., Sommer, J. A., Proctor, R. A. & Bertics, P. J. (2003) **J. Endotoxin Res.** **9**, 256-263.
49. de Rooij, J., Zwartkruis, F. J. T., Verheijen, M. H. G., Cool, R. H., Nijman, S. M. B., Wittinghofer, A. & Bos, J. L. (1998) **Science** **396**, 474-477.

**FIGURE LEGENDS**

**Fig. 1.** Expression of P2 receptors in monocytes and the effect of their activation on cAMP signaling and membrane potential. **A** and **B**, RT-PCR analyses performed using total RNA obtained from CD14+ monocytes. **C**, Monocytes were treated with nucleotides or 30 µM forskolin (as indicated) for 15 min. cAMP was then quantified by radioimmune assay after acetylation. Data are means ± SD of triplicate measurements and are representative of two independent experiments. **D**, Typical recording showing the effect of ATP (10 µM) application under whole-cell current-clamp conditions. Note that a transient depolarization precedes the hyperpolarization. **E**, Application of 10 µM UTP under the same conditions. **F**, RT-PCR products obtained after using primers for the β-subunits (KCNMB1-4) and α-subunit (KCNMA1) of BKCa channels (upper gel). The lower gel shows that IKCa (KCNN4) channels but not SKCa (KCNN1-3) channels were expressed.

**Fig. 2.** Ca2+ signaling induced by extracellular nucleotides. **A**, Application of 10 µM ATP to a single monocyte induces a rapid increase in [Ca2+] which is followed by a slower secondary increase. UTP (10 µM) elicited a similar Ca2+ transient (inset). **B**, In Ca2+-free solution, application of 10 µM ATP induces a single monotonic Ca2+ transient, whereas a"secondary increase" follows the introduction of Ca2+.
to the external solution. The latter signal is consistent with Ca\(^{2+}\) influx via store-operated channels and the inset shows that it could be reproduced by treating the cells with thapsigargin (500 nM) and intermittently switching to Ca\(^{2+}\)-free solution (indicated by solid bars). C, Plot of [ATP] versus peak intracellular Ca\(^{2+}\). D, UTP was equally effective as ATP at mobilizing Ca\(^{2+}\) from internal stores.

**Fig. 3.** ATP induces pseudopodia retraction and migration without phosphorylating Akt. A, DIC-image of a monocyte before application of ATP (x-y dimensions of frame, 18 x 19 µm). B, The same monocyte 2-3 min after application of 10 µM ATP in Ca\(^{2+}\)-free solution at 37 °C. Arrows have been added to indicate morphological changes (see also the Supplemental Movie). C, Migration assays were performed in modified Boyden chambers with various concentration of ATP in the lower well as indicated. FMLP was used as a positive control. D, Western blot analysis was performed using antibodies specific for total Akt or phosphorylated Akt (P-Akt).

**Fig. 4.** Various P2Y receptor agonists and forskolin, but not UTP, suppress LPS-induced TNF\(\alpha\) production by monocytes. A, Monocytes were treated with various concentrations of ATP\(\gamma\)S or UTP either in the absence or presence of LPS stimulation as indicated. After 16 h, concentrations of TNF\(\alpha\) in the culture supernatants were measured in duplicate by ELISA. B, ATP\(\gamma\)S (and ADP\(\beta\)S) decreased TNF\(\alpha\) production by LPS-stimulated monocytes in the presence or absence of the P2Y\(_1\) receptor blocker MRS2179. BzATP potently decreased LPS-signaling. C, Forskolin (100 µM) suppressed LPS-induced TNF\(\alpha\) production, albeit less effectively than 10 µM or 100 µM ATP\(\gamma\)S. D, The PKA blocker H89 (10 µM) did not abolish the inhibitory effect of ATP\(\gamma\)S on LPS-induced TNF\(\alpha\) production. E, Quantitative PCR analysis. All of the above data are mean ± SD and values are representative of three independent experiments.

**Fig. 5.** P2Y receptor agonists suppress LPS-induced MCP-1 production but augment IL-10 production. A, ATP\(\gamma\)S alone had no effect on MCP-1 production whereas ATP\(\gamma\)S and, to a lesser extent, 100 µM forskolin inhibited LPS-induced production of MCP-1. B, The inhibitory effect of ATP\(\gamma\)S on LPS-induced MCP-1 production was not reversed by either 100 µM 8-p-SPT or 10 µM H89. Like ATP\(\gamma\)S, BzATP also inhibited LPS-induced MCP-1 production. C, ATP dose-dependently synergized with LPS to increase IL-10 production. D, ADP\(\beta\)S, in the absence or presence of MRS2179 (P2Y\(_1\) receptor blocker), also augmented IL-10 production induced by LPS. Finally, 100 µM 8-p-SPT (adenosine receptor blocker) did not block the synergistic effect of ATP\(\gamma\)S on LPS-induced IL-10 release. In all cases, data are means ± SD of duplicate or triplicate determinations and are representative of at least three independent experiments.

**Fig. 6.** ATP\(\gamma\)S is a negative regulator of proinflammatory TLR2/6 and TLR2 signaling. A, The TLR2/6 ligand MALP-2 (100 pg/ml) induced TNF\(\alpha\) secretion by monocytes. This effect was inhibited by ATP\(\gamma\)S. B, ATP\(\gamma\)S also strongly inhibited TNF\(\alpha\) production induced by the TLR2 ligand LTA (1000 ng/ml). C, Inhibition of MALP-2-induced MCP-1 production by ATP\(\gamma\)S. D, Inhibition of LTA-induced MCP-1 production by ATP\(\gamma\)S. E, ATP\(\gamma\)S synergized with MALP-2 to increase secretion of the anti-inflammatory cytokine IL-10. F, IL-10 production stimulated by LTA was also augmented by ATP\(\gamma\)S. Data are means ± SD of duplicate determinations and are representative of at least two independent experiments.

**Fig. 7.** ATP\(\gamma\)S does not inhibit MCP-1 production stimulated by IFN-\(\gamma\) or H1N1 virus infection, and it does not decrease cell viability. A, IFN-\(\gamma\) (10 U) stimulates MCP-1 production which is not inhibited but, rather, augmented by ATP\(\gamma\)S. B, MCP-1 production induced by H1N1 virus infection is not inhibited by ATP\(\gamma\)S. C, H1N1 virus infection impairs cell viability. D, ATP\(\gamma\)S and LPS do not decrease cell viability, as assessed by the MTT assay. Data are means ± SD of duplicate determinations and are representative of at least two independent experiments.

**Fig. 8.** Proposed model in which ATP acts via P2Y\(_{11}\) receptors and increased cAMP to inhibit the proinflammatory immune response evoked by TLRs, thereby preventing excessive host tissue damage.
Figure 1
**Figure 2**

A. 10 µM ATP

B. 10 µM ATP and 0 Ca + EGTA

C. EC₅₀ = 0.12 µM

D. EC₅₀ = 0.11 µM
Figure 3

Panel A: Control ATP

Panel B: γS

Panel C: Specific migration (x1000 cells)

Panel D: Western blot showing activation of Akt and P-Akt with ATP treatment.
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
"Host tissue damage" signal ATP promotes non-directional migration and negatively regulates Toll-like receptor signaling in human monocytes

Andreas Kaufmann, Boris Musset, Sven H. Limberg, Vijay Renigunta, Rainer Sus, Alexander H. Dalpke, Klaus M. Heeg, Bernard Robaye and Peter J. Hanley

J. Biol. Chem. published online July 19, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M505301200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2005/08/02/M505301200v1.DC1

Supplemental material:
http://www.jbc.org/content/suppl/2005/08/02/M505301200.DC1