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Extracellular ATP Acting at the P2X7 Receptor Inhibits Secretion of Soluble HLA-G from Human Monocytes

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Bacterial LPS induces the release of ATP from immune cells. Accumulating evidence suggests that extracellular ATP participates in the inflammatory response as a proinflammatory mediator by activating the inflammasome complex, inducing secretion of cytokines (IL-1, IL-18) and cell damaging agents such as oxygen radicals, cationic proteins, and metalloproteases. It is not known whether ATP can also act as a proinflammatory mediator by inhibiting production of molecules down-modulating the immune response. Here, we show that extracellular ATP impairs in an IL-10-dependent fashion the expression of the tolerogenic soluble and membrane-bound HLA-G Ag in human monocytes. The effect of ATP was mimicked by BzATP (3′-O-(4-benzoylbenzoyl)-ATP) and greatly reduced by pretreatment with oATP (periodate-oxidized ATP), KN-62 (1-[N,O-bis(5-isouquinoline-sulfonyl)-N-methyl-1-tyrosyl]-4-phenylpiperazin), and an anti-P2X7 mAb, thus pointing to a specific role of the P2X7 receptor. The effect of ATP was time- and dose-dependent and was not due to a decrease in expression of IL-10 receptor. Inhibition by ATP was reverted by supplementation of culture medium with exogenous IL-10. Due to the well-known immunosuppressive activity of IL-10 and soluble HLA-G, this novel effect of ATP might be relevant for the pathophysiology and therapy of inflammatory disorders. The Journal of Immunology, 2009, 183: 4302–4311.

HLA-G Ags are currently defined as nonclassical HLA class Ib molecules, differing from classical HLA-A, -B, and -C class I molecules for the reduced allelic polymorphism (8), a restricted tissue distribution, and the occurrence of alternative mRNA splicing generating membrane-bound (HLA-G1–G4) or soluble (HLA-G5–G7) HLA-G isoforms (9). The HLA-G Ags were first observed in cytrophoblast cells (10), suggesting a functional role for these molecules in the induction and maintenance of maternal tolerogenic conditions toward the semiallogenic fetus. HLA-G production was detected in physiological conditions in the thymus and in CD14+ peripheral blood monocytes following activation of cells by IL-10, IFNs, and hormones (11, 12). Further investigations confirmed that HLA-G Ags, mainly in their HLA-G1 membrane-bound and soluble isoforms generated by proteolytic shedding (sHLA-G1) or alternative splicing (HLA-G5), mediate inhibition of NK and CD8+ lymphocyte cytotoxic activity, but also affect CD4+ functions and dendritic cell maturation.

Recently, several investigations have shown abnormal HLA-G expression in autoimmune diseases, cancer, virally infected cells, and transplanted organs, confirming the hypothesis of a tolerogenic role for these HLA class Ib molecules (13–19). In particular, increased sHLA-G levels in cerebrospinal fluids have been associated with the absence of clinical activity in multiple sclerosis (20), while in solid organ transplantation high sHLA-G serum concentrations were detected in patients with a reduced number of rejection episodes (19). A reduced sHLA-G production was suggested to play a role in the persistence of inflammatory conditions in asthma (21). However, HLA-G expression may also mediate negative clinical outcome in cancer where increased membrane-bound or sHLA-G molecules have been associated with poor prognosis (17). These observations suggest that maintenance of physiological levels of sHLA-G relates to the delicate equilibrium that regulates the inflammatory/antiinflammatory response.

The immunomodulatory effect of ATP strongly depends on the nucleotide concentration: low extracellular ATP levels induce a Th2-skewing dendritic cell phenotype and are immunosuppressive.
(22, 23), while high levels trigger the release of IL-1 family cytokines, as well as that of IL-6 and TNF-α (24–26), and are therefore proinflammatory. The strong immunostimulatory effect of elevated ATP concentrations may be mediated by release of proinflammatory cytokines or reactive oxygen species or by inhibition of production of immunosuppressive cytokines (23, 27).

In the present report we investigated the effect of ATP on sHLA-G production induced by LPS-activated human monocytes. These data show that ATP acts as a potent inhibitor of soluble and membrane-bound HLA-G production via down-regulation of IL-10 release, thus further supporting the role of ATP as a proinflammatory mediator.

Materials and Methods

Reagents

Ficoll was obtained from Cederlane Laboratories; Iscove’s medium was from Biochrom; CD hybridoma Ags and RPMI 1640 medium were from Invitrogen; LPS and KN-62 (1-(N(O-bis(5-isouquinoline-sulfonfonyl)-N-methyl-1-tetraylo)-4-phenylpiperazine)4 were purchased from Calbiochem-Novabiochem; PPADS (pyridoxal-phosphate-6-azophenyl-2’,4’-disulfonic acid) was from Sigma-Aldrich; anti-human IL-10 mAb was from MAbtech; anti-human CD14-FITC-conjugated and CD14-PE-conjugated Abs (Dako); anti-dinitrophenyl hapten mAb and anti-acid) was from Sigma-Aldrich; anti-human IL-10 mAb was from BD Biosciences; recombinant IL-10 was from PeproTech; anti-human CD14-FITC-conjugated and CD14-PE-conjugated Abs (Dako) was from Gyntens) and anti-dinitrophenyl hapten mAb and anti-acid) was from Sigma-Aldrich; and the anti-human P2X4, L4 mAb was provided by Dr. James S. Wiley (University of Sydney, Penrith Hospital, Penrith, Australia).

PBMC cultures

Informed consent for blood drawing was obtained from twenty blood donors. PBMCs were isolated by Ficoll centrifugation and suspended at the concentration of 1 x 10⁶/ml in Iscove’s medium containing 10% FCS plus antibiotics (penicillin/streptomycin) and maintained at 37°C in a humidified atmosphere with 5% CO₂. The supernatants were centrifuged, to eliminate cellular debris, and stored at −80°C.

Isolation of CD14⁺ monocytes

Monocytes were obtained from peripheral blood of healthy volunteers. Peripheral mononuclear cells were separated by Ficoll gradient centrifugation. Cells were further separated with anti-CD14 mAb-coated microbeads using MACS single-use separation columns from Miltenyi Biotech. To compare the sHLA-G secretion in PBMCs and monocyte cultures, the monocytes were suspended at the concentration of 1 x 10⁶/ml corresponding to the average monocyte concentration (10%) present in 1 x 10⁷/ml PBMC cultures. The cells were cultured in RPMI 1640 medium containing 10% FCS, 1% glutamine, 50 IU/ml penicillin, and 50 IU/ml streptomycin, and maintained at 37°C in a humidified atmosphere with 5% CO₂.

PBMC culture stimulation

To find optimal LPS concentrations and incubation times we ran preliminary dose-dependency curves with PBMCs from four healthy subjects. PBMCs were cultured as described above and stimulated with four different LPS concentrations (2.5, 5.0, 10.0, and 20.0 ng/ml). The optimal sHLA-G release occurred after a 48-h incubation in the presence of 25 ng/ml LPS (see also Ref. 28). This experimental protocol was chosen to investigate the effect of ATP on IL-10 and sHLA-G levels in culture supernatants. Increasing ATP concentrations (0.025, 0.05, 0.1, 0.5, 1 mM) were added to the PBMC cultures to inhibit sHLA-G secretion. As the optimal concentration turned out to be 1 mM, all experiments were performed with this ATP concentration unless otherwise indicated. In some experiments, LPS- and ATP-treated cultures were supplemented with 20 ng/ml recombinant IL-10 to restore sHLA-G secretion (28). The P2X4 receptor blockers were used at the following concentrations: KN-62, 50 nM; PPADS, 200 μM; and oATP, 600 μM. The anti-P2X4, mAb was used at a concentration of 10 μg/ml. The indifferent anti-dinitrophenyl hapten mAb was used at a concentration of 7.5 ng/ml. The anti-human IL-10 mAb was used at 7.5 ng/ml.

Mild acid treatment

Mild acid treatment was performed by resuspending the cells in 0.2 ml of 0.2 M citrate-phosphate buffer (pH 3.0) supplemented with 0.1% BSA (29). After a 1-min incubation on ice the sample was neutralized by adding an excess of cold 0.1% BSA in PBS (pH 7.2), centrifuged, and rinsed with PBS (pH 7.2).

Cyt fluorometric assay

Quantiﬁcation of apoptotic and necrotic cells was performed with the annexin V-FITC apoptosis detection kit from Bender MedSystems. PBMCs were centrifuged at 300 x g for 15 min, resuspended in 1 x binding buffer (10 mM/Na HEPES (pH 7.4), 140 mM/L NaCl, 2.5 mM/L CaCl₂), and stained with annexin V-FITC and propidium iodide. Analysis was conducted with a FACSCount flow cytometer (BD Biosciences) using standard settings, and the data analysis was performed with the CellQuest software (BD Biosciences). Unstained cells were classified as “live”; cells stained with annexin V only were classiﬁed as “apoptotic”; cells stained with both annexin V and propidium iodide were classiﬁed as “end-stage apoptotic/necrotic”. CD14⁺ cell viability and concentration in peripheral blood were evaluated by cell ﬂuorometry (FACS Vantage; cell ﬂuorometry), using propidium iodide staining and an anti-human CD14-PE-conjugated mAb. IL-10Rα expression was evaluated by cell ﬂuorometry, using an anti-human IL-10R-PE-conjugated Abs. HLA-G membrane expression was assessed with a MEM-G9-FITC-conjugated mAb, specific for HLA-G H chain β₂microglobulin-associated, and MEM-G1 mAb, specific for HLA-G free H chain molecule. FITC- or PE-conjugated rabbit anti-mouse IgG Abs (Dako) were used as isotype control.

Cell viability assessment

Cell viability was evaluated by trypan blue uptake or lactate dehydrogenase (LDH) release as previously described (30).

JEG-3 cell culture

Human chorioncarcinoma trophoblastic cells (JEG-3) were used as positive control, as they constitutively express HLA-G Ags (31). These cells were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FCS and maintained at 37°C in a sterile humid atmosphere under 5% CO₂ and 95% O₂.

mRNA preparation

Total cellular RNA was prepared from monocytes and JEG-3 cultures with TRIzol reagent (Invitrogen) following producer protocol. The mRNA samples were digested with DNase. The quality and quantity of mRNA samples have been assessed by a 1% agarose gel electrophoresis, followed by ethidium bromide staining. mRNA samples were immediately used for cDNA synthesis or stored frozen at −80°C until use.

mRNA amplification and analysis

Two micrograms of mRNA, prepared as previously described, was reverse transcribed for each sample using the SuperScript first-strand synthesis system (Invitrogen) according to the manufacturer’s instructions. β-actin was used as control for the mRNA content and JEG-3 cell mRNA as positive HLA-G control. Specific primers for HLA-G molecules (32) were used for HLA-G mRNA amplification. Amplification products were analyzed by ethidium bromide-stained agarose gel electrophoresis. Densitometric mRNA analysis was performed with GeneTools software in a GelImager 600 system (PerkinElmer).

ELISA assay for sHLA-G (sHLA-G1/HLA-G5)

sHLA-G1, obtained from the proteolytic cleavage of membrane-bound HLA-G1, and HLA-G5, which was later generated by mRNA alternative splicing, were assayed in PBMC and monocyte culture supernatants in triplicate as reported in the Essen Workshop on sHLA-G quantification (33). Briefly, 96-microwell plates (Nunc-ImmuNo Plate; Polysorp) were coated with the capture mAb MEM-G9 (Exbio), which recognizes shed sHLA-G1, a β₂microglobulin-associated form obtained from the proteolytic cleavage of membrane-bound HLA-G1, and the secreted HLA-G5 isofrom generated by alternative splicing. The mAb was used at the concentration of 20 μg/ml in 0.1 M carbonate buffer (pH 9.5). After a 1-h incubation at 37°C followed by overnight incubation at 4°C, plates were...
saturated with 100 μl of PBS containing 4% BSA and further incubated overnight at 4°C. Thereafter, 50 μl of undiluted samples were added to triplicate wells. Standards were run on each plate in the 1–35 ng/ml range. Dilution buffer was the negative control (blank). After incubation for 2 h at 37°C, plates were rinsed three times with PBS-Tween and incubated for 1 h at 37°C together with 50 μl of the detecting anti-β2-microglobulin HRP-conjugated mAb (Dako). Samples were rinsed five times with PBS-Tween, 50 μl of o-phenylenediamine peroxidase substrate (Sigma-Aldrich) added to each well, and incubation was carried out for 15 min at room temperature. The concentration of sHLA-G1/HLA-G5 was measured as the mean of triplicate cultures by absorbance at 450 nm with an ELISA microplate reader 400 (Packard), after subtraction of negative control OD values. Standard calibration curves were generated with supernatants from transfected HeLa-G5 cells (provided by Dr. E. Weiss, Institut für Anthropologie und Genetik, Ludwig-Maximilians-Universität, München, Germany), which were cultured in CD hybridoma AGt medium (Invitrogen) added with 1% FCS and antibiotics. Culture supernatants were collected at cell confluence and concentrated by lyophilization procedure. Following depletion of albumin with an albumin depletion kit (Enchant life science kit; Pall), the purification of the sHLA-G proteins was conducted as previously described (34). The intra-assay coefficient of variation was 1.4%, and the inter-assay coefficient of variation was 4%. The limit of sensitivity was 1 ng/ml.

**ELISA assay for HLA-G5**

HLA-G5 molecules, generated by mRNA alternative splicing, were assayed in PBMC and monocyte culture supernatants in triplicate as reported in the Essen Workshop on sHLA-G quantification (33). This ELISA test differs from the ELISA assay for sHLA-G1/HLA-G5 for the capture mAb 5A6G7 (Exbio), a specific Ab for the intron-4-derived polypeptide sequence of HLA-G5, and for the detection anti-HLA class I W6/32 (Dako) biotinylated Ab. The intra-assay coefficient of variation was 2.0%, the inter-assay coefficient of variation was 3.5%. The limit of sensitivity was 1.0 ng/ml.

sHLA-G1 was expressed as the difference between sHLA-G1/HLA-G5 and HLA-G5 concentrations.

**IL-10 concentration measurements**

IL-10 concentration was determined in triplicate undiluted samples using the commercially available human IL-10 BioSource ELISA kit with a detection limit of 0.2 pg/ml.

**FIGURE 1.** Extracellular ATP inhibits secretion of sHLA-G from LPS-activated PBMCs. A, PMBCs were cultured in Iscove’s medium at 37°C for 48 h and left untreated (Cont) or incubated in the presence of 10 ng/ml LPS (LPS) or LPS plus 1 mM ATP (LPS + ATP). Supernatants were then withdrawn and assayed by ELISA for sHLA-G content, as reported in Materials and Methods. B, Dose dependence of ATP on sHLA-G secretion. Cells were incubated in the presence of LPS plus increasing ATP concentrations. After 48 h, supernatants were withdrawn and assayed for sHLA-G as reported before.

**FIGURE 2.** Inhibition of sHLA-G release by ATP is not due to the cytotoxic effect of the nucleotide. A, PMBCs were left untreated (Cont) or incubated in the presence of 10 ng/ml LPS (LPS) or LPS plus 1 mM ATP (LPS + ATP), and at the end of the incubation, cell viability was fluorometrically analyzed as reported in Materials and Methods. Data are expresses as dot plots. Percentage of apoptotic (upper right) and late apoptosis/necrotic cells (lower right) are shown. B, LDH test was performed as reported in Materials and Methods. C, The cells were also stained with trypan blue to evaluate the cell count. Left panel, Phase-contrast images of cultured PBMCs stained with trypan blue. Few PBMCs stained with trypan blue were observed. Scale bar, 20 μm. Right panel, Alive cell percentage (open bars) and dead cells (filled bars). D, The PMBCs were stained with annexin V-FITC-conjugated (FL1) and CD14-PE-conjugated (FL2) mAbs and the percentage of CD14+ gated cells was evaluated with or without ATP treatment.
Statistics

Statistical analysis was performed with a StatView software package (SAS Institute). sHLA-G and IL-10 levels were analyzed by Mann-Whitney U test. Statistical significance was assumed for \( p > 0.05 \) (two-tailed).

Results

**ATP inhibits production of soluble HLA-G molecules from LPS-activated PBMCs**

Whether extracellular ATP might trigger release of immuno-suppressive factors is still a debated issue (22). Fig. 1A shows that under basal conditions, that is, in the absence of added stimuli, sHLA-G concentration in PBMCs supernatants was below the detection limit of the assay, while LPS addition caused a strong sHLA-G release. Incubation in the presence of LPS and ATP fully abrogated sHLA-G release (\( p < 0.0042 \) for LPS vs LPS plus ATP). To rule out possible toxic effects of ATP, we performed an ATP dose dependency (Fig. 1B) and FACS analysis to assess cell death. The ATP inhibitory effect was dose-dependent with half-maximal dose at an ATP concentration of \( \sim 200 \) \( \mu \)M and maximal effect at 1 mM. Annexin V and
propidium iodide staining (Fig. 2A) show that after a 48-h incubation in the presence of LPS plus ATP, 84.7% of the cells were vital, while 5.6% were apoptotic (Fig. 2A, upper right) and 9.7% were necrotic (Fig. 2A, lower right). This percentage of live cells did not significantly differ from that of LPS-treated (83.7%) or untreated control cells (79.1%), confirming that ATP-dependent inhibition of sHLA-G release was not due to cell death. Cell viability was also assessed by LDH release (Fig. 2B) and trypan blue exclusion (Fig. 2C). Furthermore, to rule out selective deletion of monocyte cells as a consequence of the ATP treatment, CD14+ cells were evaluated by cytofluorometry (Fig. 2D). Even though PBMCs are sensitive to the cytotoxic effect of extracellular ATP, this effect is mitigated by incubation in culture medium, especially if calf serum is present, and all these controls provided a confirmation of no cell death induced by ATP treatment. As PBMCs express multiple P2 subtypes, we tested the effect of other nucleotides.

Fig. 3A shows data obtained with two nucleotide concentrations (500 μM, black bars, or 1 mM, open bars). except for BzATP (3-O-(4-benzoyl)benzoyl-ATP), which was used at a concentration of 100 μM (black bars) or 5000 μM (open bars). ADP, which is a preferential agonist at P2Y1, P2Y12, and P2Y13 receptors, UTP, which is a preferential agonist at P2Y2 and P2Y4, UDP, a preferential agonist at P2Y6, and UDP-glucose, a preferential agonist at P2Y4, were inactive, while BzATP, which is a preferential, although not exclusive, agonist at P2X7, was a potent inhibitor at concentrations as low as 100 μM. The strong effect of BzATP, and the lack of activity of all other nucleotides tested besides ATP, strongly suggested an involvement of the P2X7 receptor. To test this hypothesis, PBMCs were preincubated in the presence of the noncompetitive covalent P2X2 inhibitor αATP, the competitive P2X2 antagonist KN-62 (35), or the widely used blocker PPADS.

Fig. 3B shows that αATP induced a complete (with BzATP as an agonist) or near complete (with ATP as an agonist) reversal of inhibition of sHLA-G secretion. KN-62 produced an ~50–60% reversal of ATP or BzATP inhibitory effect, while PPADS was ineffective on ATP or BzATP inhibitory effect. To further support the role of the P2X7 receptor we used the specific L4 mouse anti-human P2X7 mAb raised against the extracellular domain of the P2X7 receptor. This mAb was previously shown to have a potent blocking effect of P2X7 function (36, 37). Addition of the L4 mAb caused an almost complete reversal of ATP inhibitory effect, while an indifferent mAb was without effect.

Inhibition of sHLA-G secretion is not due to adenosine accumulation

The ATP inhibitory effect might be due to accumulation of adenosine, a powerful immunosuppressant generated by the hydrolysis of extracellular ATP by ectonucleotidases (38). Thus, we tested the effect of the adenosine degrading enzyme adenosine deaminase (ADA) and of the adenosine receptor agonist SCH58261. Fig. 3C shows that ADA and the adenosine receptor agonist did not reverse the inhibitory effect of ATP.

Kinetics of ATP-mediated inhibition of sHLA-G secretion

We routinely determined sHLA-G release after a 48-h incubation in the presence of the various stimuli or inhibitors. We then asked if shorter incubations in the presence of ATP were effective to inhibit sHLA-G release. We selected 15 min and 16 h as intermediate time points. At time 0, PBMCs were incubated in the presence of LPS plus ATP. At time of 15 min or time of 16 h, samples were rinsed to remove ATP and supplemented again with LPS-containing incubation solution. All samples were finally collected at 48 h. Fig. 4 shows that sHLA-G secretion was reduced by 30% by an ATP pulse of 15 min, and fully abrogated by an ATP pulse of 16 h.

**FIGURE 4.** An ATP pulse is able to reduce sHLA-G secretion induced by LPS. Cells were pulsed with ATP for 15 min (ATP (15 min) + LPS) or 16 h (ATP (16 h) + LPS) then rinsed and then LPS was added. At the end of the incubation (48 h), supernatants were withdrawn and sHLA-G content was measured by ELISA.

ATP inhibits secretion of both HLA-G1 and HLA-G5 isoforms in PBMCs and monocytes

HLA-G is normally present in two isoforms identified as HLA-G1 and HLA-G5 originating from an alternative mRNA splicing (9). HLA-G1 is a membrane-bound molecule while HLA-G5 lacks the hydrophobic transmembrane domain. Fig. 5A shows that LPS causes release of both HLA-G1 (filled bars) and HLA-G5 (open bars) isoforms, although the G5 form was predominant, and ATP inhibited release of both. Monocytes, that is, CD14+ cells, are the main source of HLA-G in the blood. Therefore, we isolated monocytes from blood and verified the inhibitory effect of the nucleotide on this purified cell population. Fig. 5B shows that ATP potently inhibited HLA-G release (△), and this effect was not due to deletion of this cell population (▪; see also Fig. 2D). The ATP dose-response curve was similar to that observed in PBMCs, strongly indicating monocytes as a candidate target for the ATP-induced response also in PBMCs. Fig. 5C shows that also in purified human monocytes the great majority of the HLA-G molecules belonged to the G5 type and that ATP-induced inhibition was effective on both G5 and sG1 isoforms.

**ATP inhibits membrane expression of HLA-G molecules**

The effects of ATP on HLA-G membrane expression were analyzed by cytofluorometry with the MEM-G9 mAb, specific for β2-microglobulin-associated HLA-G H chain. Stimulation with LPS induced a strong HLA-G membrane expression, which was fully abolished by ATP treatment (Fig. 6A). To check if HLA-G molecules could be expressed as free heavy chains, cells were also stained with MEM-G1 mAb, specific for HLA-G free H chain. No staining was revealed after LPS and LPS plus ATP treatment. Positive staining with MEM-G1 was obtained treating LPS-activated monocytes with mild acid that dissociates...
β₂-microglobulin from HLA-G H chain (29). Fig. 6B shows that ATP also reduced HLA-G expression at the transcription level.

ATP-mediated suppression of sHLA-G release is due to reduced IL-10 production

Since the main stimulus for HLA-G release is IL-10, we tested whether ATP-mediated inhibition of sHLA-G release was due to inhibition of IL-10 release. Fig. 7A shows that LPS-stimulated IL-10 secretion was significantly reduced by ATP but does not formally prove that sHLA-G decrease depended on decreased IL-10 secretion. Fig. 7B shows that an anti-IL-10 mAb (●), but not an indifferent mAb (□), strongly inhibited sHLA-G release, strongly suggesting that sHLA-G release is closely dependent on IL-10 secretion. In further support of the role of IL-10, recombinant human IL-10 restored fully (0.5 mM ATP) or partially (1 mM ATP) sHLA-G production (Fig. 7C). Addition of recombinant human IL-10 was also effective in restoring membrane expression of HLA-G (Fig. 7C).

ATP-mediated inhibition of sHLA-G release is not due to a reduced surface expression of IL-10R1

Although ATP had a clear effect on IL-10 release, it could not be excluded that nucleotide also affected monocyte (i.e., CD14⁺) cells responsiveness to IL-10 by decreasing cell surface expression of the IL-10 receptor (IL-10R1). To rule out this possibility, we correlated ATP-mediated inhibition of sHLA-G and IL-10 release to IL-10R1 subunit expression and to CD14⁺ cell number.
Fig. 8 shows that while sHLA-G and IL-10 concentrations dramatically declined during a 48-h incubation in the presence of increasing ATP doses, IL-10 R1 and CD14 cells showed a negligible reduction, suggesting that ATP caused neither down-modulation of the IL-10 receptor nor deletion of monocyte cells.

**Discussion**

Release of ATP by many different cell types is a common event. Under certain conditions associated with inflammation, high ATP concentrations (100–500 μM) can accumulate into the tissue microenvironment, thus stimulating P2 receptors (4). Given the different sensitivity to ATP of cloned P2 subtypes, the wide range of ATP concentrations that might be present at inflammatory sites confers to purinergic signaling a remarkable plasticity. Extracellular ATP has a potent immunomodulatory activity that, depending on the dose, the time, and the biochemical composition of the inflammatory microenvironment, can be either proinflammatory or antiinflammatory (27). A few P2 receptors are responsible for the proinflammatory activity of ATP and other nucleotides: P2Y2 and P2Y12 mediate chemotaxis of inflammatory cells (39–41), P2Y6 supports phagocytosis (42), and P2Y11 supports differentiation of dendritic cells (43). However, there is no doubt that the key P2 receptor that is responsible for the proinflammatory effect of ATP in vitro and in vivo is P2X7. This receptor is one of the most potent triggers of the inflammasome, and thus of IL-1β processing and secretion (44), and it causes TNF release, NO (45) and O2 generation (46) and mediates inflammatory pain (47) and cell death (48).

The potent IL-1β-releasing activity of P2X7 would be by itself sufficient to justify its proinflammatory action; however, it is well...
known that most potent proinflammatory agents act by inducing a mix of responses that include stimulation of release of immunostimulating agents and inhibition of generation of immunodepressive factors, with the end result being amplification of inflammation.

The effect of ATP on IL-10 release is rather controversial, as it is reported that this nucleotide may cause either stimulation (23, 49, 50) or inhibition of IL-10 release (51), or have no effect (22). Such contrasting results are likely dependent on the different ATP doses and experimental protocols used, as there is increasing evidence that low (<100 mM) ATP concentrations down-modulate while high (>100 mM) concentrations stimulate inflammation (27). Few reports are available on the effect of ATP on the other major immunosuppressant TGF-β. A study in kidney mesangial cells showed that TGF-β secretion is stimulated by ATP (52), but no additional data are available in other cells relevant for immunity and inflammation.

One of the most potent recently characterized endogenous immunosuppressants is HLA-G. Human leukocyte Ag G is a nonclassical human class I molecule characterized by a limited polymorphism and a restricted tissue distribution with a broad inhibitory, immunosuppressive activity. High expression of HLA-G1 by the cytotrophoblast is thought to protect the fetus from the cytotoxic activity of maternal NK cells (10, 11). This hypothesis is corroborated by several studies showing alterations in blood levels of sHLA-G (sHLA-G1) and HLA-G5 in various pregnancy complications.

The immunosuppressive activity of HLA-G can also be unveiled outside the reproductive system. sHLA-G causes apoptosis of CD8+ T and NK cells, presumably via a Fas-Fasl-dependent mechanism, inhibits proliferation of allo-specific CD4+, may enhance generation of suppressor CD4+ cells, and inhibits differentiation of immature into mature dendritic cells (13–15). The intracellular pathways responsible for this inhibitory effect are poorly known. However, there is evidence for a modified nuclear translocation of NF-κB and expression of costimulatory molecules (53).

Under physiological conditions, in the absence of factors that perturb body homeostasis, tissue and blood levels of HLA-G are very low. On the contrary, several disease states such as cancer, viral infection, and transplants potently up-regulate HLA-G expression and release. In organ transplantation there is a clear correlation between increased sHLA-G serum level and a diminished number of rejection episodes (19). Moreover, in autoimmune diseases nonactive clinical conditions in relapsing-remitting multiple sclerosis patients correlates with the presence of sHLA-G in cerebrospinal fluid (20), while impairment in the production of these antiinflammatory molecules is related to persistence of severe asthma (21). Finally, high HLA-G expression in rheumatoid arthritis and ulcerative colitis suggests that this molecule might have a broader involvement in inflammation (54, 55). Overall, these data suggest that HLA-G is a widely distributed immunoregulatory molecule with a strong immunosuppressive activity. Paradoxically, one of the most potent stimuli for HLA-G release is LPS. However, the paradox is only apparent, as many proinflammatory factors also set in motion feedback, inhibitory mechanisms devised to limit the amplification of inflammation.

In view of its potent proinflammatory action, we postulated that ATP should depress rather than stimulate IL-10 secretion. Interestingly, not only our prediction was fulfilled, but we found that the inhibitory effect of ATP extended to other potent endogenous immunosuppressants of HLA-G. The effect is dramatic, as in the presence of 1 mM ATP, LPS-stimulated soluble and membrane-bound HLA-G/β2-microglobulin-associated production was fully obliterated, while IL-10 release was decreased by ~50%. Although ATP did not fully block IL-10 release, we think that inhibition of HLA-G was due to depression of IL-10 secretion, as supplementation of the ATP-treated cultures with recombinant IL-10 fully restored HLA-G expression. This may suggest that a threshold IL-10 concentration is necessary to efficiently trigger HLA-G release. We also wondered whether the ATP effect might at least in part be due to down-modulation of the IL-10 receptor. However, analysis of ATP-treated monocytes by cytofluorometry showed no reduction in plasma membrane expression of this receptor. Another possible reason for the sHLA-G decrease might be a reduction in the overall CD14+ cell population. This does not seem to be the case, as in the different subjects the extent of CD14+ cell decrease caused by an ATP dose (1 mM) that fully abrogated sHLA-G shedding ranged between 20% and 25%. Thus, cell death does not seem to be a cause for ATP-dependent inhibition of LPS-stimulated sHLA-G production. Finally, restoration by exogenous IL-10 of HLA-G production strongly suggests that the ATP effect was not irreversible.

These observations validate the paradigm stemming from several previous studies in the field that support a chief function for...
ATP as a negative modulator of inflammation at low concentrations, an effect mainly mediated via P2Y receptors, and on the other hand a strong proinflammatory effect for ATP at high concentrations, very likely acting at the low-affinity P2X receptor (27, 56). An observation made in microglia adds a further level of complexity to this picture. In this cell type, LPS-stimulated release of IL-10 was shown to be dependent on the release of ATP and the ensuing autocrine/paracrine stimulation via P2Y receptors (57). Since LPS causes release of small ATP amounts, this observation is in keeping with the tolerogenic activity of low doses of extracellular ATP and supports data showing IL-10 releasing activity of exogenous ATP.

In conclusion, our data add further support to the hypothesis that extracellular ATP is a crucial immunomodulating agent.

Disclosures

The authors have no financial conflicts of interest.

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