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ATP suppression of interleukin-12 and tumour necrosis factor-α release from macrophages

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1 Immune cell activation releases ATP into the extracellular space. ATP-sensitive P2 purinergic receptors are expressed on immune cells and activation of these receptors alters immune cell function. Furthermore, ATP is metabolized by ectonucleotidases to adenosine, which has also been shown to alter cytokine production. In the present study, we investigated how extracellular ATP affects interleukin (IL)-12 and tumour necrosis factor (TNF)-α production in bacterial lipopolysaccharide (LPS)-treated murine peritoneal macrophages and we also examined whether extracellular ATP alters the production of the T helper 1 cytokine interferon (IFN)-γ.

2 Pretreatment of the peritoneal macrophages with ATP or various ATP analogues decreased both IL-12 and TNF-α production induced by LPS (10 μg ml^{-1}). The effect of ATP was partially reversed by cotreatment with adenosine deaminase (0.1–1 u ml^{-1}), suggesting that the suppressive effect of ATP on cytokine production is, in part, due to its degradation products.

3 Immunoneutralization with an anti-IL-10 antibody demonstrated that although ATP increases IL-10 production, the inhibition of IL-12 and TNF-α production is independent of the increased IL-10.

4 The effect of ATP was pretranslational, as it suppressed steady state levels of mRNAs for IL-12 (both p35 and p40).

5 In spleen cells stimulated with either LPS (10 μg ml^{-1}) or anti-CD3 (2 μg ml^{-1}) antibody, ATP suppressed, in a concentration-dependent manner, the production of IFN-γ.

6 These results suggest that extracellular ATP has multiple anti-inflammatory effects and that release of ATP into the extracellular space may play a role in blunting the overactive immune response in autoimmune diseases.

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Abbreviations: Ab, antibody; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; α,βMeATP, α,β-methyleneadenosine 5′-triphosphate; 2MeSATP, 2-methylthioadenosine 5′-triphosphate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; oATP, periodate-oxydized ATP; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid tetrasodium; RPA, RNase protection assay; Th, T helper

Introduction

Several lines of evidence indicate that extracellular ATP is an important modulator of immune cell function (Di Virgilio, 1995; Haskó & Szabó, 1998; Smith *et al*., 1998). This purine is released in the vicinity of immune cells from a variety of sources including the immune cell itself (Ferrari *et al*., 1997a; Sperlágh *et al*., 1998), the sympathetic nervous system (White & McDonald, 1990), and other cell types subjected to metabolic stress (Dubyak & Moatassim, 1993; Bodin & Burnstock, 1998). On the surface of immune cells, ATP interacts with P2-purinoceptors, which can be classified into P2Y (G-protein coupled) and P2X (ion channel) receptors. To date five P2Y receptors, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ and seven P2X receptors, $P2X_{1-7}$ have been cloned and pharmacologically characterized (Ralevic & Burnstock, 1998). Extracellular ATP is not stable, it is readily degraded by ectonucleotidases to adenosine, which in turn is metabolized by adenosine deaminase to inosine (White & McDonald, 1990). Both adenosine and inosine have been demonstrated to have immunomodulatory effects (Haskó & Szabó, 1998; Haskó *et al*., 1999). Adenosine is known to bind four different types of G-protein coupled cell surface molecules, termed the A_1, A_{2a}, A_{2b}, and A_3 adenosine receptors (Ralevic & Burnstock, 1998).

Interleukin (IL)-12 is a heterodimeric cytokine produced mainly by antigen-presenting cells and plays a key role in deciding the nature of immune response to exogenous or endogenous antigens. IL-12 is comprised of two disulfide-linked protein subunits designated p35 and p40, which are encoded by two different genes (Trinchieri, 1995; Gately *et al*., 1998). IL-12 is an important intermediary between innate and adaptive immunity, as it is secreted upon stimulation of monocytes/macrophages and dendritic cells and activates interferon (IFN)-γ production, proliferation, and cytolytic activity of natural killer cells and T lymphocytes (Trinchieri, 1995). In turn, IFN-γ stimulates IL-12 production and macrophage activation, which provides the foundation of an autoregulatory positive feedback loop resulting in a strong immune/inflammatory response directed against the antigen. IL-12 also plays a critical role in dictating the development of T helper (Th)1 versus Th2 cell differentiation characterized by an increased production of IFN-γ and IL-2 (Th1 cytokines) and decrease in IL-4, IL-5 and IL-10 (Th2 cytokines) formation (Trinchieri, 1995).

While IL-12 is critical for the removal of intracellular pathogens such as certain bacteria and viruses, overproduction

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of this cytokine is involved in the induction of the pathophysiology of several autoimmune diseases including multiple sclerosis (Leonard *et al*., 1995), inflammatory bowel disease (Neurath *et al*., 1995), insulin dependent diabetes mellitus (Trembleau *et al*., 1995), glomerulonephritis (Kitching *et al*., 1999) and rheumatoid arthritis (Germann *et al*., 1995). The overproduction of IL-12 is also an important pathogenic factor in inflammatory states such as septic shock (Wysocka *et al*., 1995) and the generalized Shwartzman reaction (Ozmen *et al*., 1994).

Because of the crucial role of IL-12 in orchestrating the immune response, it is important to identify endogenous mechanisms and pathways that regulate the production of this cytokine (Haskó & Szabó, 1999). In the present study, we demonstrate that ATP is an endogenous inhibitor of IL-12 expression. Furthermore, we show that ATP also decreases the production of another proinflammatory cytokine, tumour necrosis factor (TNF)-α, whereas it enhances the release of the anti-inflammatory cytokine IL-10.

Methods

Drugs and reagents

ATP, ADP, UTP, UDP, α,β-methyleneadenosine 5′-triphosphate (α,βMeATP), 2-methylthioadenosine 5′-triphosphate (2MeSATP), adenosine deaminase, and LPS (*Escherichia coli* 055 : B5) were purchased from Sigma (St. Louis, MO, U.S.A.). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). The monoclonal antibody (Ab) against mouse IL-10 and its control Ab were purchased from R&D (Minneapolis, MN, U.S.A.) and the monoclonal Ab against CD3 was obtained from Pharmingen (San Diego, CA, U.S.A.).

Preparation and treatment of peritoneal macrophages

Male BALB/c mice (25 g) were injected intraperitoneally with 2 ml of 2% thioglycollate and peritoneal cells were harvested 3–4 days later. The cells were plated on 96-well plastic plates at 1 million cells ml^{-1} and incubated in RPMI 1640 (Life Technologies (Grand Island, NY, U.S.A.) supplemented with 10% foetal bovine serum (Life Technologies, Grand Island, NY, U.S.A.) for 2 h at 37°C in a humidified 5% CO_2 incubator. Non-adherent cells were removed by rinsing the plates three times with 5% dextrose in phosphate buffered saline. Cells were treated with various concentrations of purinergic agonists or adenosine deaminase 30 min before the addition of 10 μg ml^{-1} LPS for 24 h in the continued presence of agonists or adenosine deaminase. The purinergic drugs were dissolved in media. Supernatants for IL-12, TNF-α and IL-10 determination were taken at 24 h after LPS. IL-12, TNF-α and IL-10 were determined by ELISA as described below. Cells in 6-well plates were treated the same way as in the case for cytokine protein measurements and RNA was isolated 3 h after LPS treatment.

Preparation and treatment of spleen cell suspensions

Spleens from BALB/c mice were removed aseptically, and single spleen cell suspensions in RPMI 1640 were obtained by passage through a nylon mesh (70 μM). Red blood cells were lysed using Tris-NH_4Cl (50 mM, pH 8.0). Cells were cultured in 24-well plates at 5 million cells ml^{-1} and treated with ATP followed by LPS (10 μg ml^{-1}) or anti-CD3 Ab (2 μg ml^{-1}) 30 min later for 5 days. The ATP added on day 0 was present throughout the 5 days. The plates were then centrifuged and IFN-γ was measured from the supernatants by ELISA as described below.

Cytokine assays

Cytokine concentrations in the supernatants were determined by ELISA kits that are specific against murine cytokines. Levels of TNF-α, IL-10, IL-12 (p40), IL-12 (p70), and IFN-γ were measured using ELISA kits purchased from Genzyme Co. (Boston, MA, U.S.A.). Plates were read at 450 nm by a Spectramax 250 microplate reader from Molecular Devices (Sunnyvale, CA, U.S.A.). Detection limits were 5 pg ml^{-1} for TNF-α, 0.15 pg ml^{-1} for IL-10, 10 pg ml^{-1} for IL-12 (p40), 5 pg ml^{-1} for IL-12 (p70), and 5 pg ml^{-1} for IFN-γ. Assays were performed as described previously (Haskó *et al*., 1998) and according to the manufacturer's instructions.

RNA isolation and RNase protection assay

Macrophage monolayers were washed with PBS and total cellular RNA was extracted from each well using a guanidinium isothiocyanate/chloroform based technique (TRIZOL; Life Technologies, Grand Island, NY, U.S.A.), followed by isopropanol precipitation. The RNase protection assay (RPA) was performed using a kit obtained from Pharmingen (San Diego, CA, U.S.A.). Briefly, antisense RNA multi-probe set was synthesized by *in vitro* transcription of mouse cytokine template set (mCK2) using T7 RNA polymerase in the presence of [α-^{32}P]-UTP (specific activity 3000 Ci mM^{-1}, Amersham, Arlington Heights, IL, U.S.A.). The probe set (3×10^5 c.p.m. μl^{-1}) was hybridized with target RNA (10 μg) at 56°C overnight in a total of 10 μl of hybridization buffer. The free probe and single stranded target RNA were digested with RNase at 30°C for 45 min. The proteins were digested by treating with proteinase K for 15 min at 37°C and extracted with phenol : chloroform : isoamyl alcohol (25 : 24 : 1). The aqueous phase was removed and the protected RNA precipitated with ethanol. The pellet was washed with 90% ethanol, air dried, and resuspended in 4 μl of loading buffer. The sample was then heated for 3 min at 90°C and separated on 6% sequencing gel (NOVEX, San Diego, CA, U.S.A.). The gel was then vacuum dried, and exposed to X-ray film (Kodak) for 24 h at −70°C.

Measurement of mitochondrial respiration

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondria-dependent reduction of MTT to formazan (Haskó *et al*., 1996). Cells in 96-well plates were incubated with MTT (0.5 mg ml^{-1}) for 60 min at 37°C. Culture medium was removed by aspiration, and cells were solubilized in DMSO (100 μl). The extent of reduction of MTT to formazan within cells was quantitated by measurement of optical density at 550 nm (OD_{550}) using a Spectramax 250 microplate reader.

Statistical evaluation

In the case of cytokine measurements, for each experiment the data were the mean of three wells and the results represent the mean ± s.e.mean of *n* experiments. Statistical analysis of the data was performed by one-way analysis of variance followed by Dunnett's test, as appropriate.

Results

Effect of various ATP analogues on IL-12 and TNF-α production by LPS-stimulated peritoneal macrophages

Stimulation for 24 h with LPS (10 μg ml^{-1}) induced the release of high concentrations of both IL-12 (p40) and TNF-α from peritoneal macrophages (Figure 1a,b). On the other hand, the dimeric form of IL-12 (p70) was released only at very low levels (see below). This latter finding is not surprising as the production of p40 exceeds the production of p70 by from 40 fold to more than 500 fold depending on the experimental system (Wysocka *et al*., 1995; Snijders *et al*., 1996; Haskó *et al*., 1998). Although ATP-pretreatment of the cells suppressed the production of IL-12 p70 (11.92 pg ml^{-1} in control samples vs non-detectable in 1–300 μM ATP-treated samples), the very low amounts of IL-12 p70 released did not allow for a detailed investigation on the pathways involved. Therefore, in the subsequent experiments, we measured IL-12 p40 levels to investigate the receptor subtypes and pathways involved. Pretreatment of the cells with ATP analogues suppressed both IL-12 and TNF-α production (Figure 1a,b). UTP and UDP also suppressed the production of both IL-12 and TNF-α, with EC_{50}s over 50 μM (not shown). Cell viability was not affected by any of the agonists as determined by the MTT assay (data not shown).

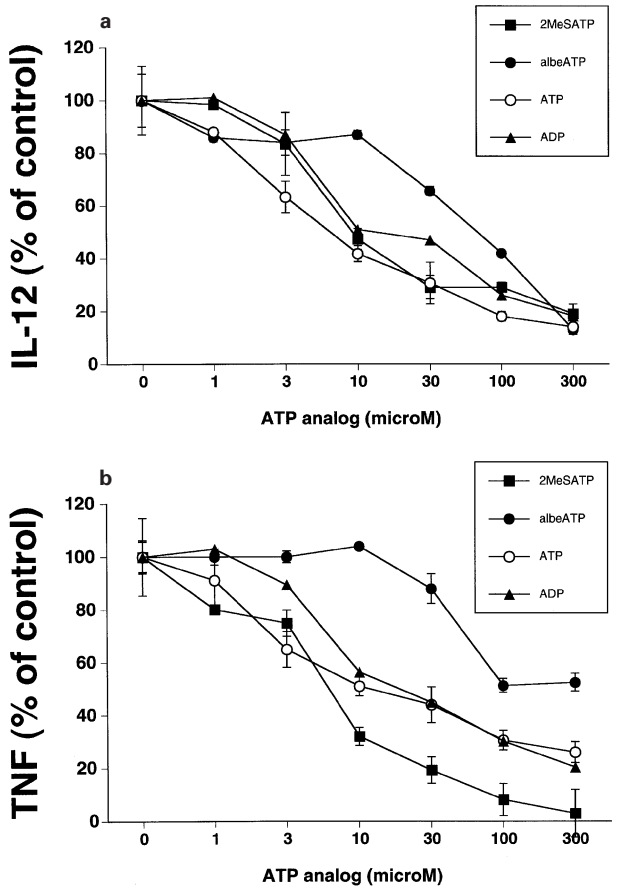

Figure 1 ATP analogues inhibit IL-12 p40 (a) and TNF-α (b) production in peritoneal macrophages stimulated with LPS (10 μg ml^{-1}). In response to LPS stimulation, the production of IL-12 and TNF-α increased from undetectable levels to 4.9±0.3 ng ml^{-1} and 32.9±1.9 ng ml^{-1}, respectively. IL-12 and TNF-α were measured from the supernatant 24 h after stimulation. Data are expressed as mean±s.e.mean from $n=2$ experiments (first, the average of three wells in each experiment was calculated and then this value was used for calculating the mean±s.e.mean of the two different experiments shown in the figure).

Because extracellular ATP is readily metabolized to adenosine, we examined the possibility that the suppressive effect of ATP on cytokine release is due to adenosine production. To eliminate the adenosine produced by the degradation of ATP, we cotreated the cells with adenosine deaminase, which deaminates adenosine to its metabolite, inosine. Adenosine deaminase in the absence of ATP caused a moderate enhancement of the production of both IL-12 and TNF-α (Table 1), which is probably due to the fact that endogenous adenosine released from the immune cells suppresses the production of proinflammatory cytokines (Haskó & Szabó, 1998). On the other hand, adenosine deaminase substantially, albeit not completely prevented the suppressive effect of ATP on both IL-12 and TNF-α production (Table 1), suggesting that the effect of ATP is, at least in part, due to the production of adenosine.

ATP augments IL-10 production, but the inhibition of IL-12 and TNF-α is independent of IL-10

ATP (100 μM) enhanced IL-10 production (417±21 pg ml^{-1} in the control samples vs 866±50 pg ml^{-1} in the ATP-treated samples; $n=4$ in both groups; $P<0.005$). Since IL-10 is known to suppress both IL-12 and TNF-α (D'Andrea *et al*., 1993), we hypothesized that the effect of ATP on IL-12 and TNF-α is due to an indirect mechanism involving the enhancement of IL-10. To investigate this possibility, we first confirmed, using an IL-10 Ab, that endogenous IL-10 inhibits IL-12 and TNF-α production; both IL-12 and TNF-α concentrations were significantly higher in the IL-10 Ab treated cells than in the control cells treated with the isotype control Ab (Figure 2a,b). While the anti-IL-10 Ab completely neutralized IL-10 activity

Table 1 Effect of P2 purinocepter antagonists on TNF-α and IL-12 levels in peritoneal macrophages

Treatment	*TNF-α* (ng ml^{-1})	*IL-12* (ng ml^{-1})
Control	5.13±0.36	2.79±0.1
100 μM ATP	0.78±0.14***	1.35±0.1***
0.1 u ml^{-1} adenosine deaminase	6.14±0.22	3.01±0.13
1 u ml^{-1} adenosine deaminase 10 μm	6.77±0.58	2.97±0.05
0.1 u ml^{-1} adenosine deaminase+100 μM ATP	2.6±0.3**	1.72±0.06***
1 u ml^{-1} adenosine deaminase+100 μM ATP	4.4±0.4	2.16±0.18*

Peritoneal macrophages were pretreated with ATP, adenosine deaminase, or the combination of ATP and adenosine 30 min before LPS-treatment. Supernatants were harvested 24 h after LPS treatment. Data are expressed as mean±s.e.mean from $n=3$ experiments (first, the average of three wells in each experiment was calculated and then this value was used for calculating the mean±s.e.mean of the three different experiments shown in the table). *Indicates $P<0.05$; **indicates $P<0.01$; ***indicates $P<0.005$ as compared to the control.

in both the ATP treated and control cells (not shown), ATP inhibited IL-12 (Figure 2a; 13±3% of control in the control antibody-treated samples and 17±4% of control in the anti-IL-10 Ab treated cells) and TNF-α (Figure 2b; 44±5% of control in the control antibody-treated samples and 46±2% of control in the anti-IL-10 Ab treated cells) production to the same extent in the presence of both the IL-10 Ab and the isotype control Ab (Figure 2a,b). Therefore, it can be concluded that the inhibition of both IL-12 and TNF-α production by ATP is independent of its stimulatory effect on IL-10.

Effect of ATP on IL-12 p35, IL-12 p40, and IL-10 mRNA levels

In order to ascertain whether inhibition of IL-12 and enhancement of IL-10 protein secretion observed with ATP receptor stimulation was at the pretranslational level, steady state mRNA levels were determined under similar *in vitro* conditions as described above. First, we determined that mRNAs for both IL-12 (p35 and p40) and IL-10 were induced by LPS as early as 3 h after stimulation (Figure 3). Pretreatment of peritoneal macrophages with ATP (10 to 300 μM, 30 min before LPS) resulted in a concentration-dependent suppression of mRNA expression for both IL-12 p35 and p40 (Figure 3). On the other hand, the level of IL-10 mRNA was only enhanced at 300 μM ATP. Thus, the molecular level of IL-12 inhibition by ATP appears to be pretranslational.

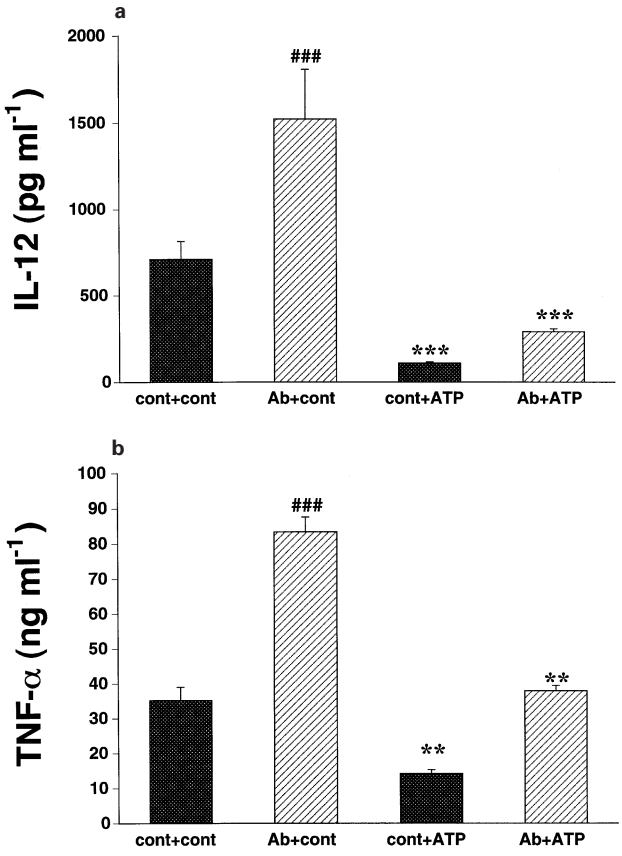

Figure 2 ATP inhibits IL-12 (a) and TNF-α (b) production to the same extent in the presence of both control Ab and anti-IL-10 Ab (both 25 μg ml^{-1}). ***, Indicates the effect of ATP (100 μM) in the presence and absence of the anti-IL-10 Ab ($P<0.05$). ###Indicates the effect of the anti-IL-10 Ab compared to the control Ab ($P<0.005$). Data are expressed as mean±s.e.mean from $n=3$ experiments (first, the average of three wells in each experiment was calculated and then this value was used for calculating the mean±s.e.mean of the three different experiments shown in the figure).

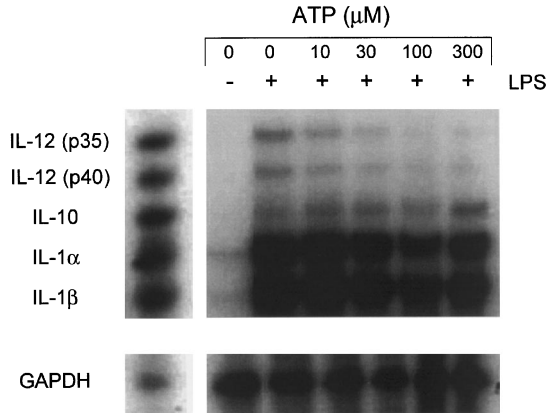

Figure 3 Effect of ATP on steady state levels of IL-10 and IL-12 p35 and p40 mRNAs. ATP causes an increase in IL-10 mRNA expression induced by LPS, while it suppresses, in a concentration-dependent manner, both IL-12 p35 and p40 mRNA levels. ATP fails to alter IL-1α, IL-1β, and GAPDH mRNA levels. Peritoneal macrophages were pretreated with ATP for 30 min, followed by an LPS treatment for 3 h. Cytokine mRNA levels were quantitated during RNase protection assay. This is representative of three different experiments.

ATP inhibits IFN-γ production in both LPS and anti-CD3-Ab stimulated spleen cells

To determine the effect of ATP on IFN-γ production, we used spleen cells, which contain both T lymphocytes and natural killer cells, the main producers of this cytokine. We stimulated the cells with LPS (10 μg ml^{-1}) or anti-CD3 Ab (2 μg ml^{-1}) and measured IFN-γ production from the supernatants 5 days after stimulation. ATP suppressed, in a concentration-dependent manner, the release of IFN-γ evoked by either LPS (Figure 4a) or the anti-CD3 Ab (Figure 4b).

Discussion

The processing and release of pro-IL-1β is highly ineffective in macrophages treated with LPS alone. However, this process can be promoted by adding ATP to the cells, which results in high levels of secreted IL-1 protein in the supernatants (Hogquist *et al*., 1991; Ferrari *et al*., 1997a). Interestingly, relatively high concentrations of ATP (1 mM) are needed to release the proinflammatory IL-1β and similarly high ATP concentrations are required to cause the activation of nuclear factor κB (Ferrari *et al*., 1997b), another ATP-induced proinflammatory effect. In contrast, in the current study, we demonstrated that low concentrations (1–300 μM) of ATP have prominent anti-inflammatory effects by suppressing IL-12, TNF-α, and IFN-γ production and by augmenting IL-10 release. The results of our study regarding the effect of ATP analogues on TNF-α production are in agreement with a previous *in vivo* study, in which intraperitoneal injection of 2MeSATP into mice decreased plasma levels of TNF-α (Denlinger *et al*., 1996). The same study also demonstrated that 2MeSATP decreases both IL-6 and IL-1 production as well as LPS-induced mortality. Although ATP has anti-inflammatory effects, it also can activate proinflammatory pathways, as it has been shown to enhance the expression of

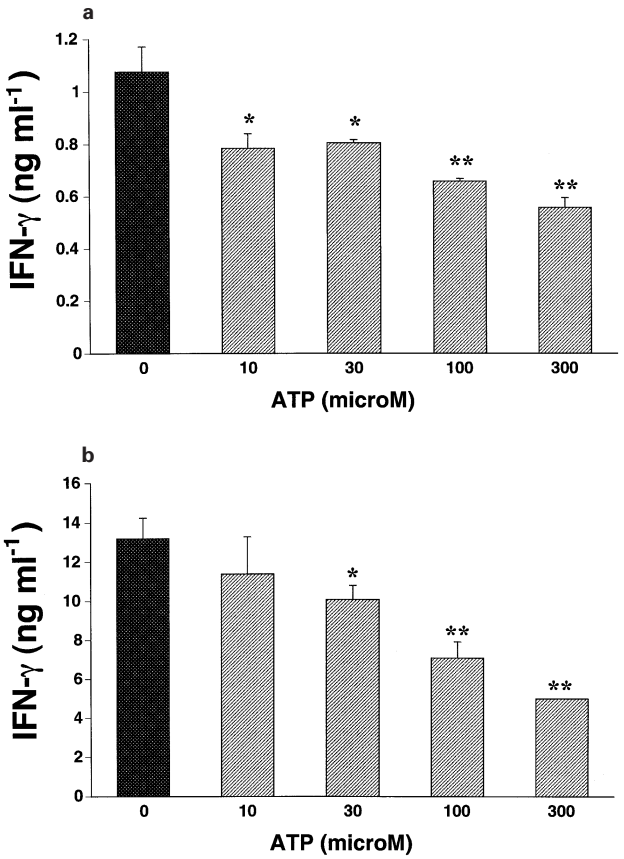

Figure 4 ATP suppresses IFN-β production by spleen cells stimulated with either LPS (10 μg ml^{-1}; (a) or anti-CD3 Ab (2 μg ml^{-1}; (b). Spleen cells were treated with ATP followed by stimulation with LPS or anti-CD-3 Ab for 5 days, and IFN-γ levels were measured from cell-free supernatants. Data are expressed as the mean$\pm$s.e.mean of $n=3$ different experiments. *Indicates $P<0.05$; **indicates $P<0.01$.

inducible nitric oxide synthase and nitric oxide production (Tonetti *et al*., 1994; Greenberg *et al*., 1997a, b; Sperlágh *et al*., 1998). However, this is not always the case, as other studies reported decreased nitric oxide synthase expression and nitric oxide production by various ATP analogues in mesangial cells (Mohaupt *et al*., 1998) or in peritoneal macrophages (Denlinger *et al*., 1996).

It is difficult to determine the receptor subtype(s) that mediate the effects of ATP on IL-12 and TNF-α production. Although ATP and its analogues are primarily recognized by P2 purinoceptors, the rapid degradation of ATP by ectonucleotides to AMP and adenosine (Haskó & Szabó, 1998) complicates the interpretation of the data. Since in our study, adenosine deaminase caused a partial reversal of the ATP effect, it can be suggested that under these conditions, the inhibitory effect of exogenously added ATP is, in part, due to the production of adenosine. The fact that adenosine deaminase failed to completely reverse the effect of ATP can be explained several ways. Firstly, it is conceivable that ATP itself inhibited cytokine production *via* stimulating P2 purinoceptors on the macrophages. The fact that α,βMeATP, a non-metabolizable P2 receptor agonist (Ralevic & Burnstock, 1998), suppressed cytokine release supports this hypothesis. Secondly, it is conceivable that adenosine deaminase at the concentrations tested (0.1–1 u ml^{-1}) was not able to convert all the adenosine molecules to inosine. Thirdly, we have recently shown that inosine itself is also capable of suppressing both IL-12 and TNF-α production in peritoneal macrophages (Haskó *et al*., 2000), suggesting that the residual immunosuppressive activity is due to inosine produced by adenosine deaminase. In summary, further studies will be necessary to clarify the receptor type(s) involved in the anti-inflammatory effects of ATP.

It is important to note that the decrease in IL-12 protein concentration coincided with a decrease in mRNA levels for both the p35 and p40 subunit, demonstrating a pretranslational regulation of IL-12 expression by ATP. Similarly, the β_2-adrenoceptor-mediated inhibition of IL-12 production is the result of decreases in both p35 and p40 mRNAs (Panina-Bordignon *et al*., 1997). These observations further support the assumption that in addition to the p40 subunit (D'Andrea *et al*., 1993), the p35 subunit is also highly regulated. Finally, the effect of ATP on IL-12 mRNA levels is not due to a general attenuation of proinflammatory cytokine mRNA accumulation, as steady state mRNA levels of the other proinflammatory cytokines IL-1α and IL-1β were not altered by ATP treatment (Figure 3). IL-10 mRNA levels were even enhanced by the highest concentration of ATP administered.

Since both IL-12 and TNF-α produced by macrophages participate in inducing IFN-γ production by natural killer cells and T lymphocytes, it is not surprising that LPS-induced IFN-γ production in spleen cell suspensions (which contain macrophages, natural killer cells, and lymphocytes) was decreased by ATP. On the other hand, the mechanism of anti-CD3-mediated IFN-γ induction is different from the one caused by LPS; anti-CD3 ligation directly stimulates IFN-γ production by T cells (Cron *et al*., 1989). Therefore, the effect of ATP in anti-CD3-stimulated spleen cells occurs *via* stimulation of purinoceptors expressed on T cells (Smith *et al*., 1998).

In summary, this study demonstrates that IL-12 and TNF-α production is suppressed by ATP and its analogues, which may be responsible for the decreased Th1 type cytokine response caused by ATP.

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