

# Adenosine inhibits IL-12 and TNF- $\alpha$ production via adenosine A<sub>2a</sub> receptor-dependent and independent mechanisms

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**ABSTRACT** Interleukin 12 (IL-12) is a crucial cytokine in the regulation of T helper 1 vs. T helper 2 immune responses. In the present study, we investigated the effect of the endogenous purine nucleoside adenosine on the production of IL-12. In mouse macrophages, adenosine suppressed IL-12 production. Although the order of potency of adenosine receptor agonists suggested the involvement of A<sub>2a</sub> receptors, data obtained with A<sub>2a</sub> receptor-deficient mice showed that the adenosine suppression of IL-12 and even TNF- $\alpha$  production is only partly mediated by A<sub>2a</sub> receptor ligation. Studies with adenosine receptor antagonists or the adenosine uptake blocker dipyridamole showed that adenosine released endogenously also decreases IL-12. Although adenosine increases IL-10 production, the inhibition of IL-12 production is independent of the increased IL-10. The mechanism of action of adenosine was not associated with alterations of the activation of the p38 and p42/p44 mitogen-activated protein kinases or the phosphorylation of the c-Jun terminal kinase. Adenosine failed to affect steady-state levels of either IL-12 p35 or p40 mRNA, but augmented IL-10 mRNA levels. In summary, adenosine inhibits IL-12 production via various adenosine receptors. These results support the notion that adenosine-based therapies might be useful in certain autoimmune and/or inflammatory diseases.—Haskó, G., Kuhel, D. G., Chen, J.-F., Schwarzschild, M. A., Deitch, E. A., Mabley, J. G., Marton, A., Szabó, C. Adenosine inhibits IL-12 and TNF- $\alpha$  production via adenosine A<sub>2a</sub> receptor-dependent and independent mechanisms. *FASEB J.* 14, 2065–2074 (2000)

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INTERLEUKIN 12 (IL-2) is a heterodimeric cytokine comprised of two disulfide-linked subunits designated p35 and p40 (1, 2). IL-12 is an important link

between innate and adaptive immunity, as it is secreted on stimulation of antigen-presenting cells and activates interferon  $\gamma$  (IFN- $\gamma$ ) production, proliferation, and cytolytic activity of natural killer cells and T lymphocytes. In turn, IFN- $\gamma$  promotes IL-12 production and macrophage activation, which provides the basis of an autoregulatory positive feedback loop resulting in a strong immune/inflammatory response directed against the antigen.

T helper lymphocytes can acquire distinct highly polarized cytokine profiles (1, 2). Early in the immune response, IL-12 also plays a critical role in directing the development of Th1 vs. Th2 cell differentiation characterized by an increased production of IFN- $\gamma$  and IL-2 (Th1 cytokines) and suppression of IL-4 (Th2 cytokine) formation. IL-12 production and a Th1 cytokine response are indispensable in the defense against intracellular pathogens (3, 4). But excessive production of IL-12 and Th1 cytokines can lead to autoimmune diseases such as multiple sclerosis (5), inflammatory bowel disease (6), insulin-dependent diabetes mellitus (7), and rheumatoid arthritis (8) as well as contribute to other inflammatory states such as septic shock (9), and the generalized Schwarzman reaction (10).

The purine nucleoside adenosine is released into the extracellular space by a wide variety of stimuli and is one of the best-characterized endogenous immunomodulator molecules. Adenosine binds to four different types of G-protein-coupled cell surface molecules, termed the A<sub>1</sub>, A<sub>2a</sub>, A<sub>2b</sub>, and A<sub>3</sub> adenosine receptors (11–13). Ligation of these receptors located on immune cells has important effects on a variety of immune cell functions including antigen presentation (14), phagocytosis (15), negative selection (16), or target cell killing (17). An important

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feature of the regulation of immune processes by adenosine is that adenosine alters immune cell production of soluble mediators, such as cytokines (14, 18–26), free radicals (20), and arachidonic acid metabolites (27). Such an interference with cell-to-cell communication within the immune system can modify the outcome of the immune response. For example, adenosine or ligands of the different adenosine receptors have been shown to ameliorate the course of a variety of immune-mediated diseases such as rheumatoid arthritis (24, 28), endotoxin shock (21, 29), nephritis (30), and uveitis (31).

Because the immune stimuli, which induce the production of IL-12, also cause the release of adenosine (32–34), it is important to explore whether and how adenosine affects IL-12 expression. In the present study, we demonstrate that adenosine, applied exogenously or released endogenously, inhibits IL-12 production.

## MATERIALS AND METHODS

### Drugs and reagents

The nonselective adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA), the selective A<sub>1</sub> receptor agonist 2-chloro-N<sup>6</sup>-cyclopentyl adenosine (CCPA), A<sub>2</sub> receptor agonist 2-p-(2-carboxyethyl) phenethyl amino-5'-N-ethyl-carboxamidoadenosine (CGS-21680), A<sub>3</sub> receptor agonist N<sup>6</sup>-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (IB-MECA), the selective A<sub>1</sub> receptor antagonists 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and 1,3-dipropyl-8-(2-amino-4-chlorophenyl)-xanthine (PACPX), A<sub>2</sub> antagonist 3,7-dimethyl-1-propargyl xanthine (DMPX), the selective A<sub>2b</sub> receptor antagonist alloxazine, A<sub>3</sub> antagonist (9-chloro-2-(2-furanyl)-5-[(phenylacetyl)amino] (1, 2, 4)-triazolo[1,5-c]quinazoline (MRS-1220), and dipyrindamole were obtained from Research Biochemicals Inc. (Natick, Mass.). The selective A<sub>2a</sub> antagonist 4-(2-[7-amino-2-(2-furyl)-[1,2,4]-triazolo[2,3-a][1,3,5]triazin-5-yl-amino]ethyl)phenol (ZM241385) was purchased from Tocris Cookson (Bristol, U.K.). Adenosine, adenosine deaminase, and lipopolysaccharide (LPS) (*Escherichia coli* 055:B5) were purchased from Sigma (St. Louis, Mo.); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Fisher Scientific (Pittsburgh, Pa.). The monoclonal Ab against mouse IL-10 and its control Ab were purchased from R&D (Minneapolis, Minn.). IFN- $\gamma$  was obtained from Genzyme (Cambridge, Mass.).

### Preparation and treatment of peritoneal macrophages

Male BALB/c and A<sub>2a</sub> receptor-deficient mice and wild-type littermates were injected intraperitoneally with 2 ml of 2% thioglycollate and peritoneal cells were harvested 3–4 days later. The generation of the A<sub>2a</sub> receptor-deficient mice was described previously (35). Near congenic mutant mice (N6 on C57Bl/6 background) and their wild-type littermates were used in this study. The cells were plated on 96-well plastic plates at 1 million cells/ml and incubated in RPMI 1640 (Life Technologies (Grand Island, N.Y.) for 2 h at 37°C in a humidified 5% CO<sub>2</sub> incubator. Nonadherent cells were removed by rinsing the plates three times with 5% dextrose in phosphate-buffered saline (PBS). Cells were treated with

various concentrations of adenosine or adenosine receptor agonists 30 min before the addition of 10  $\mu$ g/ml LPS or a combination of LPS and IFN- $\gamma$  (100 U/ml) for 24 h; supernatants for IL-12, TNF- $\alpha$ , and IL-10 determination were taken 24 h after LPS. Selective antagonists of adenosine receptors, adenosine deaminase, or dipyrindamole were added 30 min before LPS. IL-12, TNF- $\alpha$ , and IL-10 were determined by enzyme-linked immunoassay (ELISA) as described below.

### Culture and treatment of J774.1 macrophages

The mouse macrophage cell line J774.1 was grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Cells were cultured in 96-well plates (200  $\mu$ l medium per well) until 80% confluence. The cells were pretreated with various concentrations of adenosine and treated with a combination of LPS and IFN- $\gamma$  (100 U/ml) for 24 h as for peritoneal macrophages (see above).

### Cytokine assays

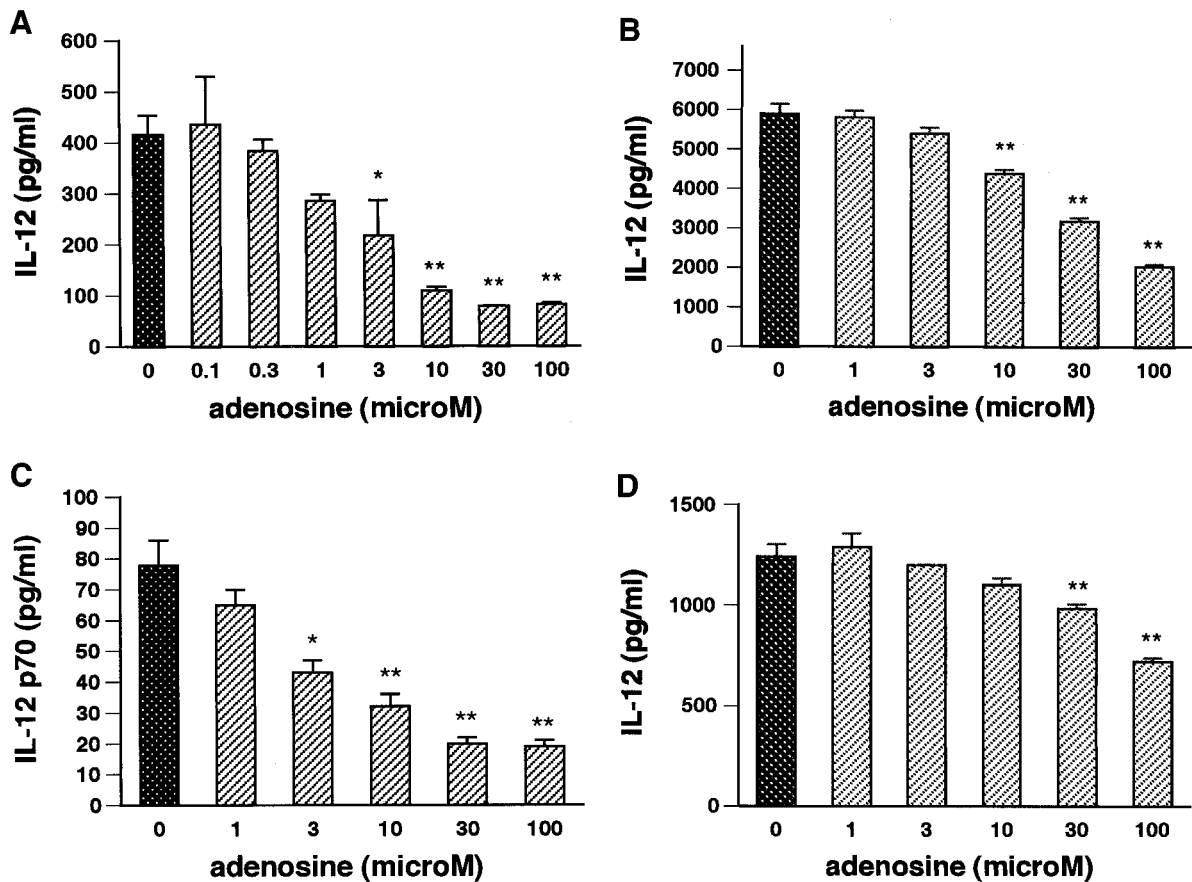
Cytokine concentrations in the supernatants were determined by ELISA kits that are specific against murine cytokines. Levels of TNF- $\alpha$ , IL-10, IL-12 (total), and IL-12 (p70) were measured using ELISA kits purchased from Genzyme Co. (Boston, Mass.). Plates were read at 450 nm by a Spectra-max 250 microplate reader from Molecular Devices (Sunnyvale, Calif.). Detection limits were 5 pg/ml for TNF- $\alpha$ , 0.15 pg/ml for IL-10, 10 pg/ml for IL-12 (total), and 5 pg/ml for IL-12 (p70). Assays were performed as described previously (20) and according to the manufacturer's instructions.

### Western blot analysis

Cells in 6-well plates were pretreated with adenosine or vehicle; 30 min later the cells were stimulated with LPS (10  $\mu$ g/ml) for 15 min. After washing with PBS, the cells were lysed by the addition of modified radioimmunoprecipitation buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.25% Na-deoxycholate, 1% Nonidet P-40, 1  $\mu$ g/ml pepstatin, 1  $\mu$ g/ml leupeptin, 1 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>). The lysates were transferred to Eppendorf tubes, centrifuged at 15,000 g, and the supernatant was recovered. Protein concentrations were determined using a Bio-Rad Protein Assay (Bio-Rad, Hercules, Calif.). Ten micrograms of sample was separated on a 8–16% Tris-glycine gel (Novex, San Diego, Calif.) and transferred to a nitrocellulose membrane. The membranes were probed with anti-phospho-mitogen-activated protein kinase (MAPK; p42/p44), anti-phospho-c-Jun amino-terminal protein kinase (JNK, Promega, Madison, Wis.), anti-phospho-p38 MAP kinase (p38 MAPK, New England Biolabs, Beverly, Mass.) and subsequently incubated with a secondary horseradish peroxidase-conjugated donkey anti-rabbit antibody (Boehringer, Indianapolis, Ind.). Bands were detected using ECL Western Blotting Detection Reagent (Amersham Life Science, Arlington Heights, Ill.).

### RNA isolation and RNase protection assay

Cells in 6-well plates were treated the same way as for cytokine protein measurements, and RNA was isolated 3 h after LPS treatment. 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), followed by isopropanol pre-



**Figure 1.** Adenosine inhibits *A*) IL-12 (total) production in peritoneal macrophages stimulated with LPS (10 µg/ml) and *B*) the combination of LPS and IFN-γ (100 U/ml). Adenosine also inhibits the production of IL-12 p70 in peritoneal cells induced with the combination of LPS and IFN-γ (100 U/ml; *C*) or p40 in J774 cells induced with LPS and IFN-γ (*D*). IL-12 was measured from the supernatants 24 h after stimulation. Data are expressed as the mean ± SE of 6 wells. \**P* < 0.05; \*\**P* < 0.01.

precipitation. The RNase protection assay was performed using a kit obtained from PharMingen (San Diego, Calif.). 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 [ $\alpha$ - $^{32}$ P] UTP (specific activity 3000 Ci/mM, Amersham). The probe set ( $3 \times 10^5$  cpm/ $\mu$ l) 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). 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 (20). Cells in 96-well plates were incubated with MTT (0.5 mg/ml) for 60 min at 37°C. Culture medium was removed by aspiration and cells were solubilized in dimethyl sulfoxide (100 µl). The extent of reduction of MTT to formazan within cells was quantitated by measurement of

optical density at 550 nm (OD<sub>550</sub>) using a Spectramax 250 microplate reader.

#### Statistical evaluation

Values in the figures, tables, and text are expressed as mean ± SE of *n* observations. Statistical analysis of the data was performed by one-way analysis of variance, followed by Dunnett's test, as appropriate.

## RESULTS

### Adenosine inhibits IL-12 production in peritoneal cells and J774 macrophages

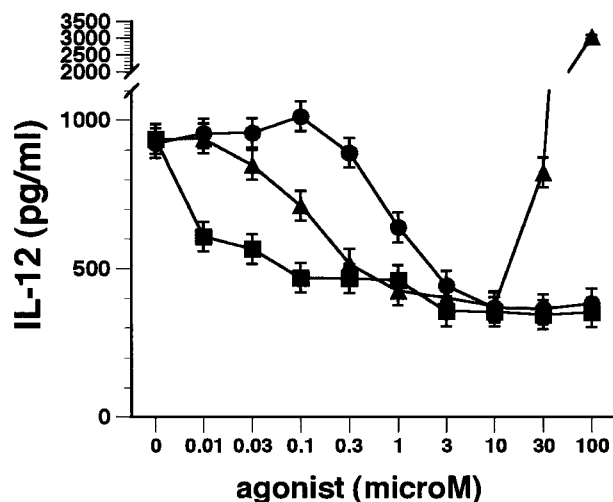
Stimulation for 24 h with LPS (10 µg/ml) induced the release of IL-12 p40 from peritoneal macrophages (Fig. 1A). Although IFN-γ alone failed to trigger the production of IL-12 (not shown), the combination of LPS (10 µg/ml) and IFN-γ (100 U/ml) resulted in a synergistic potentiation of IL-12 p40 release (Fig. 1B). This combination, but not LPS alone, evoked detectable levels of IL-12 (p70) in the peritoneal macrophages (Fig. 1C). Adenosine pre-

treatment of peritoneal macrophages stimulated with LPS caused a concentration-dependent inhibition of IL-12 p40 production (Fig. 1A). The effect of adenosine was less pronounced when IL-12 was induced with LPS/IFN- $\gamma$  (Fig. 1B, C). Adenosine was even less potent in LPS/IFN- $\gamma$ -stimulated J774 cells (Fig. 1D). Finally, neither adenosine nor the selective adenosine receptor agonists affected cellular viability as tested with the MTT assay (not shown).

### Effect of selective agonists and antagonists of adenosine receptors on IL-12 production in peritoneal cells

Figure 2 shows that the adenosine receptor agonists inhibit IL-12 production in peritoneal macrophages with the order of potency CGS-21680 > IB-MECA > CCPA, suggesting that the suppression of IL-12 by adenosine is predominantly an A<sub>2a</sub> receptor-mediated process. The effect of IB-MECA was biphasic. The inhibition of IL-12 production reached its maximum at 10  $\mu$ M, but at 30  $\mu$ M IB-MECA, IL-12 levels returned to the baseline, and at 100  $\mu$ M, IB-MECA caused a threefold increase in IL-12.

In the next step, we evaluated a series of adenosine receptor antagonists. The A<sub>2</sub> receptor antagonist DMPX augmented IL-12 production (Table 1), suggesting that endogenous adenosine suppresses IL-12 production. The effect of the nonselective A<sub>2a</sub> antagonist DMPX was mimicked by the selective A<sub>2a</sub> antagonist ZM241385 (1  $\mu$ M), whereas the selective A<sub>2b</sub> antagonist alloxazine (1, 10, and 50  $\mu$ M) had no effect (not shown). The A<sub>1</sub> antagonist DPCPX also increased IL-12, but another A<sub>1</sub> agonist, PACPX (1, 10, and 50  $\mu$ M), failed to enhance production of this



**Figure 2.** Effect of various adenosine receptor agonists on LPS-induced IL-12 production in peritoneal macrophages. The adenosine receptor agonists were added 30 min before LPS and IL-12 levels were measured from supernatants taken 24 h after LPS. Data are expressed as the mean  $\pm$  SE of 6 wells. Circles: CCPA; triangles: IB-MECA; squares: CGS-21680.

**TABLE 1.** Effect of selective adenosine receptor antagonists on IL-12 release from LPS-treated peritoneal macrophages<sup>a</sup>

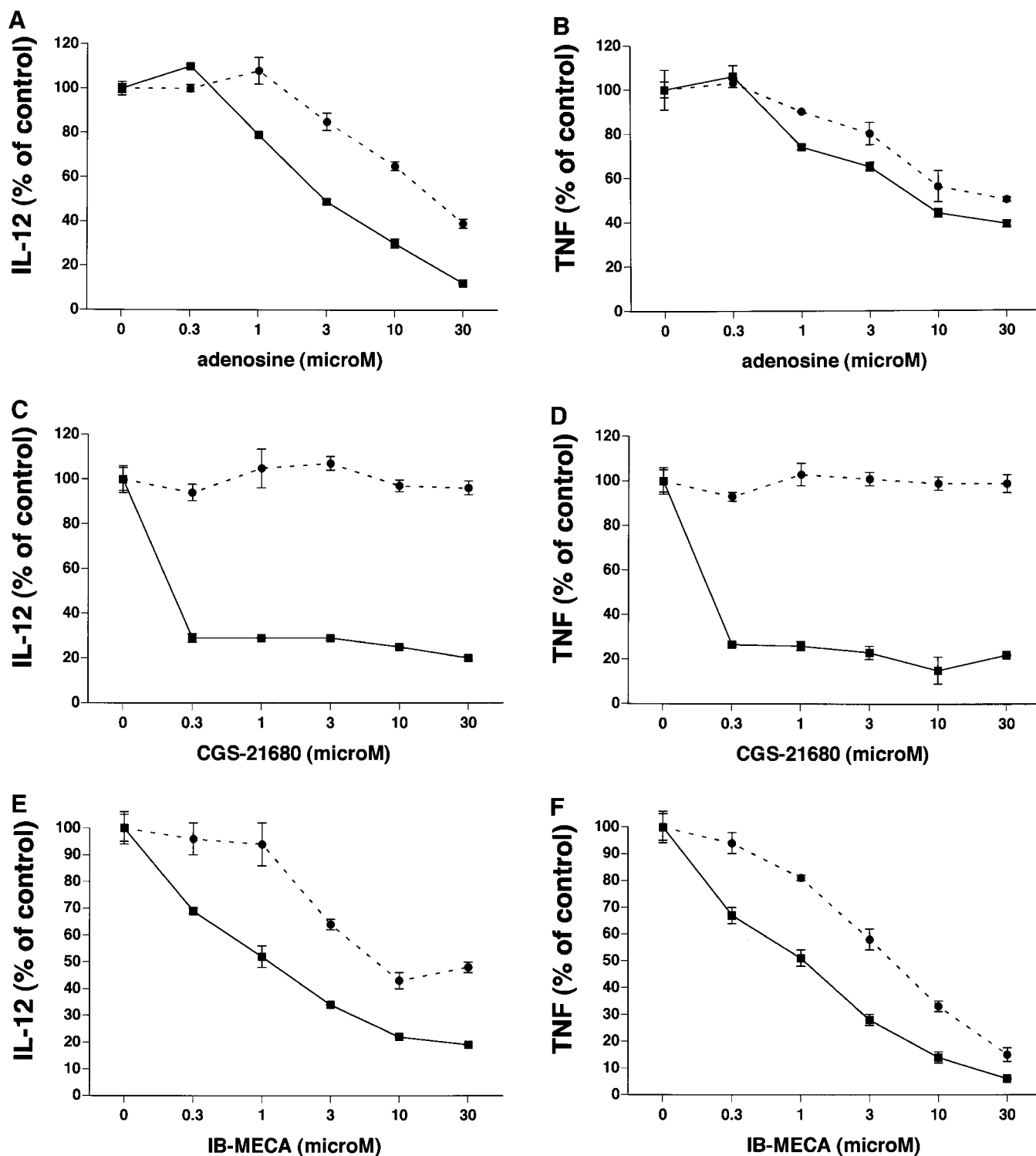
Compound	Receptor specificity	IL-12 concentration
vehicle	—	299 $\pm$ 9.59
DMPX (1 $\mu$ M)	A <sub>2</sub>	346.25 $\pm$ 38.32*
DMPX (10 $\mu$ M)	A <sub>2</sub>	374.5 $\pm$ 8.39**
DMPX (50 $\mu$ M)	A <sub>2</sub>	357.25 $\pm$ 19.28*
DPCPX (1 $\mu$ M)	A <sub>1</sub>	356.75 $\pm$ 28.4*
DPCPX (10 $\mu$ M)	A <sub>1</sub>	235.75 $\pm$ 9.99*
DPCPX (50 $\mu$ M)	A <sub>1</sub>	143 $\pm$ 11.04**

<sup>a</sup> Peritoneal macrophages were pretreated with various concentrations of adenosine receptor antagonists 30 min before stimulation with LPS (10  $\mu$ g/ml). Data are expressed as the mean  $\pm$  SEM of 6 wells. \*  $P$  < 0.05; \*\*  $P$  < 0.01.

cytokine, suggesting that the effect of DPCPX may be nonspecific. It must be pointed out that these agents alone in the absence of LPS did not induce IL-12 production; therefore, the possibility of LPS contamination can be excluded. The suppressive effect of higher concentrations of DPCPX (10 and 50  $\mu$ M) is probably due to an inhibitory effect of this xanthine compound on phosphodiesterases. The blockade of these enzymes both by xanthine and nonxanthine inhibitors has been shown to decrease IL-12 production in various *in vitro* and *in vivo* systems (36, 37). Although MRS-1220, a potent antagonist of human A<sub>3</sub> receptors, was ineffective in augmenting IL-12 production (data not shown), it cannot be excluded that endogenous adenosine via A<sub>3</sub> receptors augments the IL-12 response, because this agent is not a potent A<sub>3</sub> antagonist in rodent systems (38). Further evidence for an inhibitory role of endogenous adenosine is that the adenosine uptake blocker dipyridamole (10  $\mu$ M) decreased IL-12 production (971  $\pm$  31 pg/ml in controls vs. 718  $\pm$  25 in dipyridamole-treated;  $P$  = 0.0009), similarly to adenosine receptor agonists.

### Adenosine inhibits both IL-12 and TNF- $\alpha$ production in A<sub>2a</sub> receptor-deficient mice

Since the order of potency of adenosine receptor agonist suggested that A<sub>2a</sub> receptors are responsible for the suppressive effect of adenosine on IL-12 production, we tested the effect of adenosine in A<sub>2a</sub> receptor-deficient mice. Because CGS-21680 and IB-MECA were the most potent agonists, we also tested the effect of these agents on both IL-12 and TNF- $\alpha$  production. Although CGS-21680 completely lost its efficacy in the A<sub>2a</sub> receptor-deficient mice (Fig. 3C, D), both adenosine (Fig. 3A, B) and IB-MECA (Fig. 3E, F) continued, albeit to a lesser extent than in the wild-type littermates, to suppress both IL-12 and TNF- $\alpha$  production in the A<sub>2a</sub> receptor-deficient mice. Therefore, it can be concluded that the effect



**Figure 3.** Effects of adenosine (A, B), CGS-21680 (C, D), and IB-MECA (E, F) on IL-12 and TNF- $\alpha$  production in peritoneal macrophages stimulated with LPS (10  $\mu$ g/ml) for 24 h. Continuous lines indicate the effects of drugs in wild-type mice, whereas dashed lines represent data from A<sub>2a</sub> receptor-deficient mice.

of adenosine is only partially due to stimulation of A<sub>2a</sub> receptors.

### Receptor desensitization

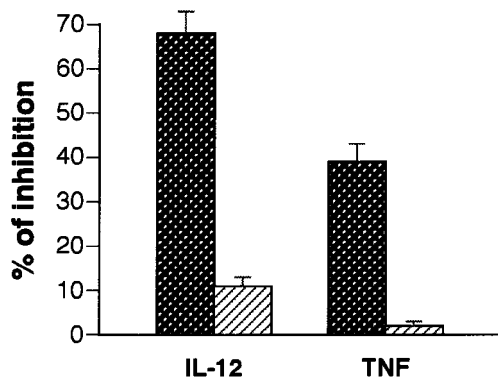
Because adenosine receptors are subject to desensitization by exposure to adenosine analogs in various

experimental systems (39), we examined whether the effect of adenosine diminishes after prolonged stimulation of adenosine receptors. For this purpose, we pretreated peritoneal cells with NECA (10  $\mu$ M), a nonmetabolizable, nonselective adenosine receptor agonist for 12 h that has been shown to desensitize adenosine receptor-mediated processes (40). **Figure**

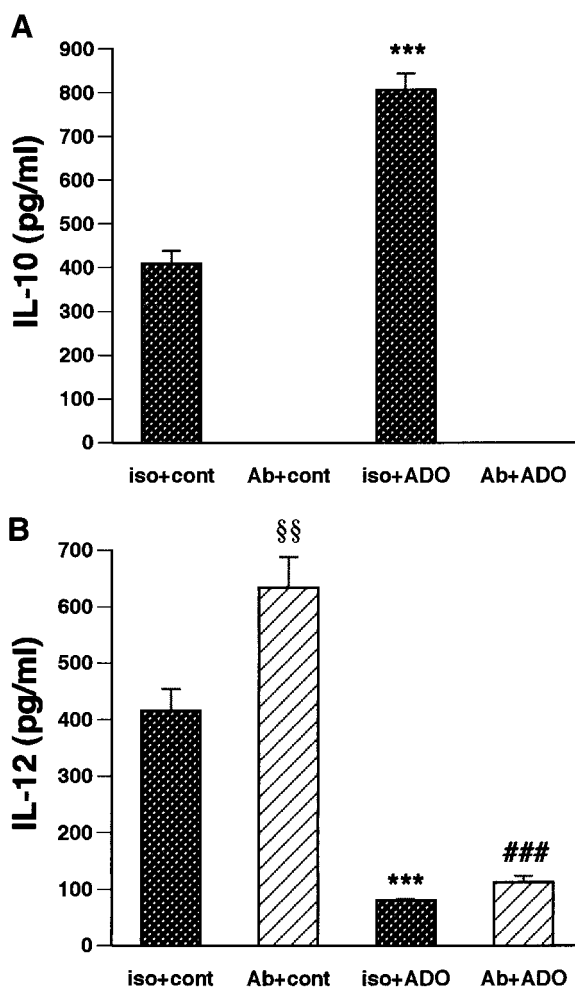
4 demonstrates that after the 12 h exposure to NECA, adenosine was no longer able to inhibit either IL-12 or TNF- $\alpha$  release, indicating that adenosine receptors on macrophages are subject to down-regulation.

### IL-10 is not involved in the suppression of IL-12 production by adenosine

Because IL-10 is one of the most important cytokines known to down-regulate IL-12 production and adenosine has been shown to augment the release of IL-10 in human monocytes (41), we examined the possibility that the effect of adenosine on IL-12 is due to its ability to increase IL-10 production. Adenosine caused a significant augmentation of IL-10 release in mouse peritoneal macrophages (Fig. 5A). By using an IL-10 Ab, we confirmed that endogenous IL-10 inhibits IL-12 production, since the IL-12 concentrations were significantly higher in the IL-10 Ab-treated cells than in the control cells treated with the isotype control Ab (Fig. 5B). Whereas the anti-IL-10 Ab completely neutralized IL-10 activity in both the adenosine-treated and control cells (Fig. 5A), adenosine inhibited IL-12 production to the same extent in the presence of both the IL-10 Ab and the isotype control Ab (Fig. 5B;  $19 \pm 2\%$  in the control samples and  $17 \pm 3\%$  in the anti-IL-10 Ab treated cells). Therefore, it can be concluded that the inhibition of IL-12 production by adenosine is independent of its effect on IL-10.



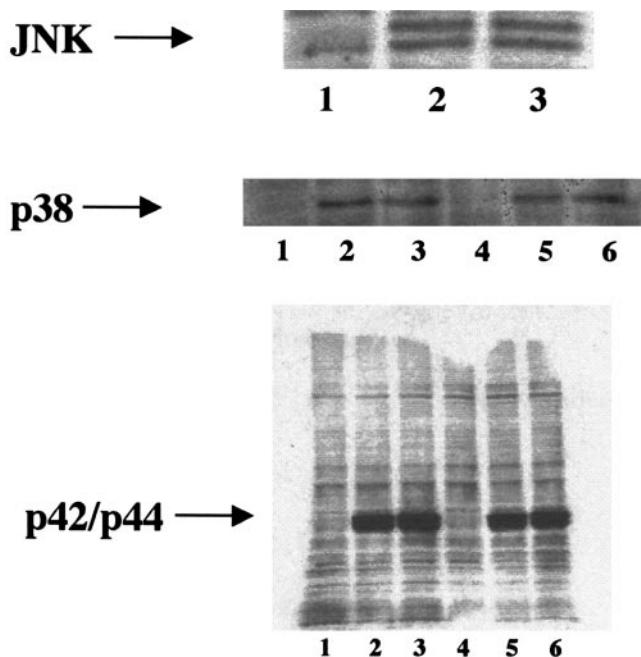
**Figure 4.** The inhibition of IL-12 and TNF- $\alpha$  production by adenosine receptor stimulation is subject to receptor desensitization. To desensitize adenosine receptors, peritoneal macrophages were pretreated with the nonmetabolizable, nonselective adenosine receptor agonist NECA for 12 h. Cells were then pretreated with adenosine (10  $\mu$ M) and stimulated with LPS (10  $\mu$ g/ml) 30 min later. Supernatants for determination of IL-12 and TNF- $\alpha$  production were taken 24 h after LPS. Dotted bars indicate the extent of inhibition without NECA pretreatment; cross-hatched bars show the extent of inhibition in the presence of NECA. Data are expressed as the mean  $\pm$  SE of 6 wells.



**Figure 5.** A) Adenosine augments IL-10 production in peritoneal macrophages. Adenosine (30  $\mu$ M) was added 30 min before LPS (10  $\mu$ g/ml) and IL-10 was measured from supernatants collected 24 h after LPS. The anti-IL-10 antibody (Ab) completely neutralizes IL-10. \*\*\*Effect of adenosine in the presence of the control (iso) antibody ( $P < 0.005$ ). B) Adenosine inhibits IL-12 production to the same extent in the presence of both control antibody and the anti-IL-10 antibody. \*\*\*The effect of adenosine in the presence of the control antibody ( $P < 0.005$ ). ###The effect of adenosine in the presence of the IL-10 antibody ( $P < 0.005$ ). §§Effect of the anti-IL-10 antibody as compared to the control antibody ( $P < 0.01$ ). Data are expressed as the mean  $\pm$  SE of 6 wells.

### Adenosine fails to influence LPS-induced activation of the p38 and p42/44 MAPKs or phosphorylation of JNK

Because activation of the MAPKs p38 and p42/44 and the phosphorylation of JNK are important pathways during macrophage activation (42), we tested the possibility that adenosine exerts its effects on IL-12 and TNF- $\alpha$  production via interfering with the activation of these enzymes. Although LPS treatment of peritoneal macrophages caused the activation of both p38 and p42/44 as well as JNK as determined 15 min after LPS treatment, adenosine administered



**Figure 6.** Adenosine fails to affect both p38 and p44 MAP kinase as well as JNK activation in mouse peritoneal macrophages (lanes 1, 4: no stimulation; lanes 2,5: LPS 10 µg/ml; lanes 3, 6: adenosine pretreatment 30 min before LPS). This is representative of 2 different experiments.

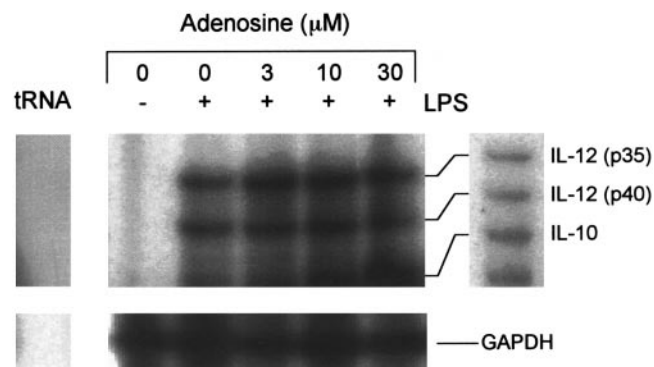
30 min before the LPS challenge failed to alter the activation of these intracellular pathways (Fig. 6).

#### Effects of adenosine on IL-12 p35, IL-12 p40, and IL-10 mRNA levels

To determine whether inhibition of IL-12 and enhancement of IL-10 protein secretion observed with adenosine receptor stimulation was at the pretranslational level, steady-state mRNA levels were determined under similar *in vitro* conditions as those described above. First, we determined that mRNAs for both IL-12 and IL-10 were induced by LPS as early as 3 h after stimulation (Fig. 7). Whereas pretreatment of peritoneal macrophages with adenosine (3 to 30 µM, 30 min before LPS) resulted in a significant increase in IL-10 mRNA at 3 h after LPS, IL-12 p35 and p40 mRNA expression was not altered by adenosine pretreatment (Fig. 7). Thus, the molecular level of IL-10 augmentation but not IL-12 inhibition by adenosine is pretranslational. This latter finding was confirmed in experiments where adenosine was shown to inhibit IL-12 production even when added to the cells 6 h after LPS (Fig. 8).

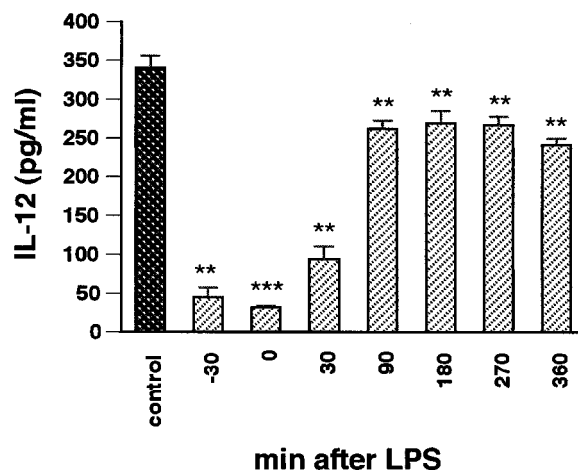
## DISCUSSION

Inflammation, ischemia, or cellular metabolic stress evoke high levels of extracellular nucleotides and



**Figure 7.** Effect of adenosine on steady-state levels of IL-10, IL-12 p35, and p40 mRNAs. Adenosine causes a concentration-dependent (3–30 µM) increase in IL-10 mRNA expression induced by LPS, whereas it has no effect on either IL-12 p35, p40, or GAPDH mRNA levels. Peritoneal macrophages were pretreated with adenosine for 30 min, followed by an LPS treatment for 3 h. Cytokine mRNA levels were quantitated using RNase protection assay. This is representative of 2 different experiments.

nucleosides, with adenosine levels reaching as high as 10 µM in the extracellular space (32, 33). This observation and the fact that exogenous adenosine, at similar levels, has substantial anti-inflammatory and anti-ischemic activity have led to the suggestion that this nucleoside is an endogenous anti-inflammatory and anti-ischemic (retaliatory) metabolite (32, 33). That IL-12 contributes to the induction of inflammatory processes has long been known (1, 2), and recently this cytokine was implicated in the pathophysiology of ischemia-reperfusion injury (43). Our results showing that adenosine inhibits IL-12 production suggest that the beneficial effects of adenosine in inflammatory processes or in ischemia-reperfusion injury can be attributed partly to its inhibition of IL-12 production. Because the immunomodulatory effects of adenosine are mediated by



**Figure 8.** The effect of adenosine on IL-12 production, when added to macrophages before or after LPS. Data are expressed as the mean  $\pm$  SE of 6 wells. \*\* $P < 0.01$ , \*\*\* $P < 0.005$ .

the ligation of various adenosine receptors, it is important to determine which receptor(s) are involved in the suppressive effect of adenosine on IL-12 production.

To characterize the receptor subtypes involved, we performed several studies. The pharmacological studies using various ligands of adenosine receptors indicated that the order of potency of selective adenosine receptor agonists was CGS-21680 > IB-MECA > CCPA and suggested that the A<sub>2a</sub> receptor was the most likely candidate for producing the inhibitory effect of adenosine on IL-12 production. Next, to help confirm a role for the A<sub>2a</sub> receptor subtype, we treated mice deficient for the A<sub>2a</sub> receptor, hypothesizing that the effect of adenosine would be lost in the A<sub>2a</sub> receptor-deficient mice. To our surprise, at concentrations higher than 1 μM, adenosine maintained its inhibitory activity in the A<sub>2a</sub> receptor-deficient mice, although the extent of inhibition was less than in the wild-type littermate mice. Because adenosine at 1 μM, but not at higher concentrations, lost its efficacy in the A<sub>2a</sub>-deficient mice, it is plausible that at lower extracellular adenosine concentrations, the A<sub>2a</sub> receptor is the only receptor involved in the inhibitory effect of adenosine on both IL-12 and TNF-α production. On the other hand, at higher adenosine concentrations, such as those that may occur in pathophysiological states associated with severe cellular distress, adenosine suppresses cytokine production via other pathways besides the A<sub>2a</sub> receptor. Similarly to adenosine, IB-MECA suppressed IL-12 and TNF-α production in both the knockout and wild-type mice, although it lost some of its efficacy in the knockout animals. This also suggests that the suppressive effect of IB-MECA has a component dependent on A<sub>2a</sub> receptors. In sharp contrast, the effect of CGS-21680 was completely abolished in the A<sub>2a</sub> receptor-deficient mice, confirming the functional inactivation of A<sub>2a</sub> receptors on the macrophages. The rank order of agonist potencies suggests that the A<sub>3</sub> receptor may be the other receptor involved in the action of adenosine. This notion is supported by the finding of a recent study in which the suppression of TNF-α by adenosine was primarily due to A<sub>3</sub> receptor activation (44).

Since adenosine receptor antagonists increased IL-12 secretion and the adenosine uptake blocker dipyrindamole decreased IL-12 release, it can be concluded that adenosine is an endogenous inhibitor of IL-12. These data are in accord with the results of a previous study in which endogenous adenosine was shown to suppress TNF-α production in human peripheral blood mononuclear cells (45). Although in our study adenosine deaminase was expected to have a similar effect as the receptor antagonists (that is, to enhance IL-12 production by decreasing the bioavailability of adenosine), this enzyme failed to

significantly enhance IL-12 production (not shown). A possible explanation for this contradiction is that the product of adenosine deamination, inosine, could offset the enhancing effect of the lower endogenous adenosine concentrations. This idea is supported by a recent study showing that inosine suppresses IL-12 production (46).

Another interesting finding is that the A<sub>3</sub> receptor agonist IB-MECA has a biphasic effect on IL-12 production in the BALB/c mice, with an inhibition of up to 10 μM but augmentation at 100 μM. It is conceivable that, similar to another A<sub>3</sub> receptor agonist, N<sup>6</sup>-(3-chlorobenzyl)-5'-N-methylcarboxamidoadenosine (47), IB-MECA at higher concentrations opens ATP-gated potassium channels, which could contribute to the enhancement of IL-12 release. In fact, our preliminary studies show that ATP-gated potassium channel openers increase IL-12 production (unpublished observation).

Adenosine receptors have been shown to be desensitized in many *in vitro* and *in vivo* studies (39). In the current study, we showed that the effect of adenosine on cytokine production is abrogated in macrophages when adenosine receptors are exposed to prolonged stimulation of the adenosine receptors by NECA. This finding has important implications for the treatment of chronic inflammatory diseases with adenosine-based anti-inflammatory therapies, as the efficacy of treatment might diminish with time. In this respect, further studies will be needed to characterize which subtypes of adenosine receptors are subject to desensitization and which subtypes are resistant to such an effect.

Since endogenous IL-10 inhibits LPS-induced IL-12 production (48) and adenosine was shown to increase IL-10 release in human monocytes (41), we hypothesized that the inhibition of IL-12 by adenosine was secondary to the enhancement of IL-10 release. Indeed, similar to what was observed in human monocytes, our data showed that adenosine up-regulates IL-10 production in mouse peritoneal macrophages. Therefore, to study the possible association between increased IL-10 and decreased IL-12 production, we examined the effect of adenosine on IL-12 production after immunoneutralization of IL-10. Our data indicate that up-regulation of IL-10 and the suppression of IL-12 production are independent processes in the current setting.

The mechanism of augmentation of IL-10 production, but not that of inhibition of IL-12 protein release by adenosine, involves an effect on the expression of mRNAs for these cytokines. In this respect, the adenosine regulation of IL-12 in mouse macrophages is similar to that of TNF-α; that is, adenosine receptor stimulation does not affect steady-state levels of TNF-α mRNA in mouse macrophage cell lines (21). On the other hand, in a recent

study using the U937 human macrophage cell line, adenosine receptor ligation suppressed TNF- $\alpha$  mRNA levels (44). Because LPS-induced activation of the MAPKs p38 and p42/44 and of JNK are important pathways that can modulate cytokine production at the posttranscriptional step (49, 50) and adenosine receptor stimulation has been demonstrated to regulate these kinases (51, 52), we evaluated whether adenosine suppression of IL-12 production could be explained by an effect on these pathways. Our results showed that the activation of none of these enzymes is altered by adenosine. Regarding the p42/44 MAPK pathway, our study confirms the results of the study of Sajjadi et al. (44) in which adenosine stimulation failed to alter MAPK activation in human macrophages.

In summary, the current study demonstrates that adenosine suppresses the production of IL-12 and TNF- $\alpha$ . These activities are brought about by stimulating A<sub>2a</sub> and other adenosine receptors. These effects may contribute to the beneficial effects of adenosine receptor agonists (34) and adenosine-releasing drugs such as adenosine kinase inhibitors (53), methotrexate (54), sulfasalazine (55), or aspirin (56) in several autoimmune and inflammatory diseases. FJ

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