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Effect of A_{2B} adenosine receptor gene ablation on adenosine-dependent regulation of pro-inflammatory cytokines.

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The abbreviations used are: A_{2B}KO, A_{2B} adenosine receptor knockout; A_{2A}KO, A_{2A} adenosine receptor knockout; ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; IL, interleukin; IPDX, 3-isobutyl-8-pyrrolidinoxanthine; LPS, lypopolysacharide; MRS 1754, 8-[4-[((4-cyanophenyl) carbamoylmethyl)oxy]phenyl]-1,3-di(n-propyl)xanthine; NECA, 5'-N-ethylcarboxamidoadenosine; RT-PCR, reverse transcription-polymerase chain reaction; TNF-α, tumor necrosis factor-alpha

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ABSTRACT

Pharmacological studies suggest that A_{2B} adenosine receptors mediate pro-inflammatory effects of adenosine. Recently, this concept has been challenged by the finding that A_{2B} knockout (A_{2B}KO) mice have moderate inflammation due to elevated basal plasma tumor necrosis factor-alpha (TNF-α) and an exaggerated response to lypopolysacharide (LPS) challenge. However, it is unclear if this phenomenon actually reflects the loss of putative taming of pro-inflammatory cytokine production via activation of A_{2B} receptors by endogenous adenosine. Here, we studied adenosine receptor-dependent regulation of interleukin (IL)-6 and TNF-α blood plasma levels in A_{2B}KO and wild-type mice *in vivo* and their release from peritoneal macrophages *ex vivo*. Stimulation of adenosine receptors with 5'-N-ethylcarboxamidoadenosine (NECA) upregulated IL-6 and suppressed LPS-induced TNF-α in wild-type mice. The selective A_{2B} antagonists IPDX and MRS 1754 inhibited NECA-induced IL-6 release but not the suppression of LPS-induced TNF-α secretion from macrophages. Genetic ablation of A_{2B} receptors abrogated NECA-induced increases in IL-6 release from mouse peritoneal macrophages and dramatically reduced the ability of NECA to raise IL-6 plasma levels *in vivo*. In contrast, the absence of A_{2B} adenosine receptors did not affect NECA-induced suppression of LPS-activated TNF-α release in macrophages, nor did it reduce the ability of NECA to suppress LPS-induced increase in TNF-α plasma levels *in vivo*. Thus, our results indicate that stimulation of A_{2B} receptors upregulates the pro-inflammatory cytokine IL-6, and argue against the recently suggested anti-inflammatory role of A_{2B} receptors in suppression of LPS-stimulated TNF-α production by adenosine.

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INTRODUCTION

There is growing evidence that adenosine plays an important role in the regulation of inflammation. Adenosine is an intermediate product of adenine nucleotide metabolism, and also serves as a signaling molecule that can modulate cellular functions via binding to cell surface G-protein-coupled receptors of the P1 purinergic family comprised of A_1, A_{2A}, A_{2B} and A_3 adenosine receptor subtypes. Among adenosine receptors, the A_{2B} subtype has the lowest affinity to adenosine. Although A_{2B} receptors are likely to remain silent under normal physiological conditions (Fredholm et al., 2001b), they can be activated during inflammation when interstitial adenosine concentrations are increased as a result of cell stress, injury, and tissue hypoxia (Fredholm et al., 2001a).

Numerous studies suggest that A_{2B} receptors are involved in adenosine-dependent regulation of pro-inflammatory paracrine factors. We have previously shown that stimulation of A_{2B} receptors in human mast cell line HMC-1 increased production of pro-inflammatory cytokines interleukin (IL)-1β, IL-3, IL-4, IL-8 and IL-13 (Feoktistov and Biaggioni, 1995; Ryzhov et al., 2004). Further studies in human primary cell cultures demonstrated that A_{2B} receptors increase MCP-1 and IL-6 release from airway smooth muscle cells and fibroblasts, thus mediating the putative pro-inflammatory and pro-fibrotic actions of adenosine in asthma (Zhong et al., 2004; Zhong et al., 2005). Pharmacological inhibition of A_{2B} receptors significantly reduces elevations in pro-inflammatory cytokines as well as mediators of fibrosis and airway destruction induced by high adenosine levels in the lungs of adenosine deaminase-deficient knockout mice (Sun et al., 2006b). Stimulation of pro-inflammatory cytokines via A_{2B} receptors is not limited to the lung; it has been also observed in various cell types of different origins. A_{2B}

receptors stimulate IL-8 production in human microvascular endothelial (Feoktistov et al., 2002) and glioblastoma (Zeng et al., 2003) cell lines. A_{2B} receptors were implicated in stimulation of IL-6 release in osteoblasts (Evans et al., 2006), intestinal epithelial cells (Sitaraman et al., 2001), pituitary folliculostellate cells (Rees et al., 2003), astrocytes (Schwaninger et al., 1997), astrocytoma (Fiebich et al., 2005) and astroglioma cells (Fiebich et al., 1996). For this and other reasons, A_{2B} receptors have been suggested to mediate pro-inflammatory actions of adenosine.

Contrary to this body of evidence, a recent report on a mouse phenotype resulting from deletion of A_{2B} receptor gene suggested that the A_{2B} receptor protects against inflammation and excessive vascular adhesion (Yang et al., 2006). This conclusion was based on low-grade inflammation observed in the A_{2B} knockout (A_{2B}KO) mice at rest manifested by increased leukocyte adhesion to the vascular wall and increased expression of adhesion molecules in vascular endothelium as consequence of elevated tumor necrosis factor-alpha (TNF-α) plasma levels. Lypopolysacharide (LPS)-induced elevations of cytokine levels were also exaggerated in A_{2B}KO mice. Bone marrow-derived cells, particularly macrophages, have been identified as a primary source of increased cytokine production in A_{2B}KO mice. Interpretation of these results as evidence of anti-inflammatory actions of A_{2B} receptors seems surprising not only because it contradicts previous pharmacological evidence, but also because a baseline inflammatory phenotype in A_{2B} receptor knockout animals assumes tonic stimulation of A_{2B} receptors, which is unlikely given their low affinity for adenosine (Linden, 2006). Alternatively, the observed changes could be interpreted as a result of developmental adaptation to the

genetic removal of the A_{2B} receptor, or could reflect the loss of a previously unrecognized function of the A_{2B} receptor protein independent of adenosine signaling.

In this context, it should be noted that the effects of direct agonist stimulation of adenosine receptors on pro-inflammatory cytokine production have not been studied in A_{2B}KO mice. Without this information, it is difficult to determine if the reported inflammatory phenotype in A_{2B}KO mice indeed reflects the loss of adenosine–dependent signaling functions of A_{2B} receptors or points toward the alternative interpretations outlined above. In the current study we chose two well-recognized immunomodulatory actions of adenosine and investigated the effects of A_{2B} receptor gene ablation on adenosine-dependent stimulation of IL-6 release and suppression of LPS-induced TNF-α secretion (Hasko et al., 1996; Ritchie et al., 1997) by directly activating adenosine receptors in wild-type and A_{2B}KO mice.

MATERIALS AND METHODS

Reagents

Lipopolysaccharide (LPS) (from *Escherichia coli*, serotype 055:B5), 5'-N-ethylcarboxamidoadenosine (NECA), and 8-[4-[((4-cyanophenyl) carbamoylmethyl)oxy]phenyl]-1,3-di(n-propyl)xanthine (MRS 1754) were purchased from Sigma (St. Louis, MO). 3-isobutyl-8-pyrrolidinoxanthine (IPDX) was synthesized as previously described (Feoktistov et al., 2001). Dimethyl sulfoxide (DMSO) was purchased from Sigma. When used as a solvent, final DMSO concentrations in all assays did not exceed 0.1% and the same DMSO concentrations were used in vehicle controls.

Mice

6- to 8-week-old, age- and sex-matched mice were used. A_{2B}KO mice were obtained from Deltagen (San Mateo, CA) and wild-type (WT) C57BL/6 mice were purchased from Harlan World Headquarters (Indianapolis, IN). Genotyping protocols for A_{2B}KO have been previously described (Csoka et al., 2007). All of the A_{2B}KO mice used in these studies were back-crossed to the C57BL/6 genetic background for 10 generations.

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

Real-time RT-PCR analysis was performed as previously described (Ryzhov et al., 2007). Total RNA was isolated from cells using the RNeasy Mini kit (Qiagen, Valencia, CA). Real-time RT-PCR was carried out on ABI PRISM 7900HT Sequence Detection System (PE Applied Biosystems, Foster City, CA). Primer pairs and FAM-labeled probes for murine adenosine receptors and β-actin were provided by Applied Biosystems. RT-PCR reactions utilizing 1 μg of DNase-treated total RNA were

performed under conditions recommended by the manufacturer. A standard curve for each amplicon was obtained using serial dilutions of total RNA. The results from triplicate polymerase chain reactions for a given gene at each time point were used to determine mRNA quantity relative to the corresponding standard curve. The relative mRNA quantity for a given gene measured from a single reverse transcription reaction was divided by the value obtained for β-actin to correct for fluctuations in input RNA levels and varying efficiencies of reverse transcription reactions.

Measurement of cAMP accumulation

Cyclic AMP accumulation was measured as previously described (Feoktistov et al., 2002). Cells in 12-well plates were pre-incubated in 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 g/L D-glucose, 10 mM HEPES-NaOH, pH 7.4 and 1 U/mL adenosine deaminase containing the cAMP phosphodiesterase inhibitor papaverine (1 mM) for 15 min at 37°C. Adenosine agonists, forskolin or their vehicle (DMSO) were added to cells, and the incubation was allowed to proceed for 10 min at 37°C. The reaction was stopped by the addition of 1/5 volume of 25% trichloroacetic acid. The extracts were washed five times with 10 volumes of water-saturated ether. Cyclic AMP concentrations were determined using a cAMP assay kit (GE Healthcare, Little Chalfont, UK). In parallel measurements, total protein in macrophages was determined using a Coomassie Plus Bradford™ assay (Pierce, Rockford, IL) and intracellular cAMP levels were expressed as picomoles per milligram of protein.

Analysis of IL-6 and TNF-α secretion from macrophages

Freshly isolated macrophages were incubated in the presence or absence of LPS, and in the presence or absence of NECA and A_{2B} receptor antagonists in RPMI 1640 medium with 10% calf serum, 1X Antibiotic Antimycotic mixture (Sigma) and 1 U/mL adenosine deaminase for 16 h at 37°C. After collection of culture media, the cells were lysed with 0.4 N NaOH and assayed for total protein using a Coomassie Plus Bradford™ assay (Pierce, Rockford, IL). IL-6 and TNF-α concentrations in culture media were measured using ELISA kits (R&D Systems, Minneapolis, MN). Released cytokines were expressed as picograms per milligram of protein.

Animal procedures

All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Mice were injected intraperitoneally with LPS (10 mg/kg), NECA (0.5 mg/kg) or its vehicle (DMSO) in sterile phosphate-buffered saline (PBS). NECA was injected 10 minutes before LPS. In another set of experiments, mice were injected intraperitoneally with NECA (0.5 mg/kg) or its vehicle (DMSO) in PBS without subsequent LPS injection. Blood was collected from the retro-orbital vein using heparinized Natelson blood collecting tubes (Fisher Scientific, Pittsburgh, PA) 1.5 h after LPS injection for TNF-α assay or 3 h after NECA injection for IL-6 assay, and cytokine levels were determined from the isolated plasma. These time points were chosen from ancillary studies which showed that the maximal plasma levels of TNF-α and IL-6 have been reached at 1.5 h after LPS injection and 3 h after NECA injection respectively.

Isolation of Mouse Peritoneal Macrophages

Mice were injected intraperitoneally with 2 ml of 3% thioglycollate. After 4 days, peritoneal cells collected by lavage were seeded onto 24-well plates in RPMI 1640 medium with 10% calf serum and 1X Antibiotic Antimycotic mixture (Sigma) for 2 h to allow the macrophages to adhere to the plates. Nonadherent cells were subsequently removed by washing with RPMI 1640 medium, and the adherent macrophages were used for experiments immediately following isolation.

Statistical Analysis

Data were analyzed using the GraphPad Prism 4.0 software (GraphPad Software Inc. San Diego, CA) and presented as means±SEM. Multiple comparisons between different animal groups (wild-type versus A_{2B}KO mice) were performed using one-way analysis of variance (ANOVA) followed by Bonferroni's post-tests. Comparisons between groups treated with A_{2B} receptor antagonists and a control untreated (Vehicle) group were performed using one-way ANOVA followed by Dunnett's post-tests. In studies comparing the effects of stimulation of adenosine receptors on different groups of animals, we used a two-factor ANOVA followed by Bonferroni's post-tests. Factor A was the intervention (stimulation with NECA or no stimulation), fixed, 2 levels. Factor B was the animal group (wild-type versus A_{2B}KO mice), fixed, 2 levels. Of particular interest was the interaction between the two factors; that is, whether the animal group modifies the response to NECA. A p value < 0.05 was considered significant.

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RESULTS

Effect of adenosine A_{2B} receptor gene ablation on adenosine receptor mRNA expression in peritoneal macrophages. Real-time RT-PCR analysis of wild-type macrophages revealed preferential expression of mRNA encoding A_{2A} receptor subtype (0.96±0.18% of β-actin), with lower expression of A_{2B} and A_3 receptor subtypes (0.43±0.11 and 0.3±0.05% of β-actin, respectively) and no detectable levels of A_1 receptor transcripts (Figure 1). As expected, we did not detect the expression of A_{2B} receptor mRNA in A_{2B}KO macrophages. We also documented that A_{2B} receptor gene ablation had no significant effect on A_{2A} and A_3 receptor mRNA expression in A_{2B}KO macrophages (0.88±0.2 and 0.25±0.05% of β-actin, respectively).

Effect of adenosine A_{2B} receptor gene ablation on cAMP levels in peritoneal macrophages. Adenosine receptor subtypes were initially characterized by their effects on adenylate cyclase activity; A_{2A} and A_{2B} receptors stimulate adenylate cyclase via coupling to G_s proteins, whereas A_1 and A_3 receptors inhibit stimulation of this enzyme via coupling to G_i proteins (Fredholm et al., 2001a). Therefore, we questioned whether A_{2B} receptor gene ablation has any effect on cAMP levels in peritoneal macrophages. As seen in Figure 2, comparative analysis revealed no significant difference in basal cAMP levels between macrophages isolated from wild-type and A_{2B}KO mice (0.563±0.06 and 0.689±0.036 pmol/mg, respectively; Figure 2). Similarly, we found no significant difference in adenosine receptor-mediated cAMP accumulation between macrophages isolated from wild-type and A_{2B}KO mice. Stimulation of adenosine receptors with the stable adenosine analog NECA (10 μM) increased cAMP levels in wild-type and A_{2B}KO macrophages by 56±7 and 41±9%, respectively. These effects were relatively small

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compared to more robust effects of the adenylate cyclase stimulator forskolin (1μM) which was used as a positive control (Figure 2). Thus, our results suggest that A_{2B} receptor gene ablation has negligible effect on the regulation of cAMP levels in mouse peritoneal macrophages.

Effect of adenosine A_{2B} receptor gene ablation on IL-6 secretion. Incubation of quiescent macrophages for 16 hours results in accumulation of low levels of IL-6 in cell culture media (Figure 3A). Adenosine stimulates IL-6 production in various cells including peritoneal macrophages (Ritchie et al., 1997). In agreement with previously published data, 10 μM NECA stimulated IL-6 generation in wild-type macrophages resulting in a six-fold increase in IL-6 production from 61±14 pg/mg to 361±57 pg/mg. Although A_{2B} receptor gene ablation had no effect on basal release of IL-6, it almost completely abrogated the NECA-induced IL-6 secretion from A_{2B}KO macrophages (Figure 3A). Two-way ANOVA demonstrated significant interaction (p=0.0003) between adenosine A_{2B} receptor gene ablation and NECA effects. *In vivo*, intraperitoneal injection of 0.5 mg/kg NECA to wild-type mice produced significantly higher IL-6 plasma levels (171±17 pg/ml) compared to IL-6 plasma levels (14±2 pg/ml) observed in the control group of wild-type mice injected with vehicle (0.1% DMSO in PBS). Although A_{2B} receptor gene ablation had no significant effect on IL-6 plasma levels in control mice injected with vehicle, it dramatically reduced the ability of NECA to raise IL-6 plasma levels in A_{2B}KO mice (Figure 3B). Two-way ANOVA confirmed significant (p=0.004) interaction between these factors. Thus, we concluded from these studies that A_{2B} receptors are essential for adenosine-dependent upregulation of IL-6.

Effect of adenosine A_{2B} receptor gene ablation on TNF-α secretion. In addition to stimulation of IL-6 production, adenosine is known to suppress TNF-α release induced by LPS (Hasko et al., 1996; Ritchie et al., 1997; Hasko and Cronstein, 2004; Kreckler et al., 2006). Our results showed that quiescent A_{2B}KO macrophages secrete higher levels of TNF-α compared to macrophages isolated from wild-type mice (72±4 and 43±11 pg/mg, respectively; $p<0.05$). Stimulation of adenosine receptors with 10 μM NECA had no significant effect on basal TNF-α release from both wild-type and A_{2B}KO macrophages (Figure 4A). In contrast, NECA significantly suppressed TNF-α release from both wild-type and A_{2B}KO macrophages activated by 10 ng/ml LPS (Figure 4B). However, two-way ANOVA revealed no interaction between A_{2B} receptor gene ablation and the effects of NECA ($p=0.5$). In other words, the absence of A_{2B} adenosine receptors did not change the suppression of LPS-activated TNF-α release in response to stimulation of macrophage adenosine receptors by NECA. *In vivo*, intraperitoneal injection of 0.5 mg/kg NECA to wild-type mice prior to challenging with 10 mg/kg LPS significantly attenuated TNF-α plasma levels compared to those induced by LPS alone. This inhibitory effect of NECA was even greater in A_{2B}KO mice (Figure 4C), indicating that A_{2B} receptors are not involved in adenosine-dependent suppression of LPS-induced TNF-α release.

Effect of adenosine A_{2B} receptor antagonists on IL-6 and TNF-α secretion. In a complementary approach we chose two structurally unrelated selective A_{2B} antagonists IPDX and MRS 1754 (Feoktistov et al., 2000; Ji et al., 2001) to analyze the role of A_{2B} receptors in the regulation of inflammatory cytokines in peritoneal macrophages. We initially confirmed that both IPDX and MRS 1754 significantly inhibited NECA-induced

IL-6 secretion, but had no effect on the basal cytokine release in the absence of NECA (Table 1). In contrast, both IPDX and MRS 1754 failed to antagonize NECA-dependent suppression of LPS-induced TNF-α secretion. Albeit not statistically significant, there was a tendency for both selective A_{2B} antagonists to attenuate LPS-induced TNF-α release in the absence or presence of NECA (Table 2).

DISCUSSION

Adenosine released under inflammatory conditions can exert anti-inflammatory or pro-inflammatory actions depending on the specific adenosine receptor subtype involved. The low-affinity A_{2B} receptor has been known to mediate pro-inflammatory effects of adenosine by upregulation of cytokines and growth factors. This view has been supported by a large body of evidence provided by pharmacological analysis of adenosine-dependent cytokine and growth factor secretion in various cells, tissues and organs (Feoktistov and Biaggioni, 1995; Fiebich et al., 1996; Schwaninger et al., 1997; Feoktistov et al., 2002; Zeng et al., 2003; Rees et al., 2003; Ryzhov et al., 2004; Zhong et al., 2004; Fiebich et al., 2005; Zhong et al., 2005; Evans et al., 2006; Sun et al., 2006b). This concept has been recently challenged by finding that A_{2B}KO mice exhibit moderate inflammation primarily caused by elevated basal and LPS-stimulated plasma TNF-α (Yang et al., 2006). This led the authors to suggest that the A_{2B} receptor attenuates inflammation. However, it is unclear if these results actually reflect the loss of the putative taming of pro-inflammatory cytokine production via activation of A_{2B} receptors by endogenous adenosine.

We revisited these conclusions by studying adenosine-dependent regulation of cytokine secretion in A_{2B}KO and wild-type mice. We conducted studies in peritoneal macrophages previously identified as a major source of elevated plasma TNF-α in A_{2B}KO mice (Yang et al., 2006), and *in vivo* using previously established models of adenosine receptor-dependent IL-6 secretion (Weiterova L, 2007) and suppression of LPS-induced TNF-α production (Parmely et al., 1993; Salvatore et al., 2000; Gomez and Sitkovsky, 2003).

Our data show that engagement of A_{2B} receptors by agonist leads to pro-inflammatory events that can be blocked by selective A_{2B} antagonists or by genetic ablation of A_{2B} receptors. We determined that A_{2B} receptors mediate stimulation of IL-6 production, but found that they are not involved in suppression of LPS-induced TNF-α secretion by adenosine. Stimulation of adenosine receptors with the stable adenosine analog NECA upregulated IL-6 and suppressed LPS-induced TNF-α in wild-type mice. Two structurally different selective A_{2B} antagonists IPDX and MRS 1754 both inhibited NECA-induced IL-6 release but had no significant effect on the ability of NECA to suppress LPS-induced TNF-α secretion from peritoneal macrophages. In this respect, our results correlate well with a recent work of Kreckler et al (2006), which demonstrated that the potency and efficacy of NECA to inhibit LPS-induced TNF-α secretion from wild-type mouse peritoneal macrophages are not influenced by blocking A_{2B} receptors with MRS 1754.

Genetic ablation of A_{2B} receptors completely abrogated NECA-induced increase in IL-6 release from peritoneal macrophages and dramatically reduced the ability of NECA to raise IL-6 plasma levels *in vivo*. In contrast, the absence of A_{2B} adenosine receptors did not affect adenosine receptor-dependent suppression of LPS-activated TNF-α release in peritoneal macrophages, nor did it reduce the ability of NECA to suppress LPS-induced increase in TNF-α plasma levels *in vivo*. Thus, our results obtained in A_{2B}KO mice confirm the previous concept stemming from pharmacological studies that A_{2B} receptors mediate pro-inflammatory effects of adenosine. Our data, however, do not support the hypothesis that the A_{2B} receptor mediates tonic anti-inflammatory effects of adenosine in wild-type mice by suppressing TNF-α secretion.

Mouse peritoneal macrophages express A_{2A}, A_{2B} and A_3 adenosine receptors. The dominant role of A_{2A} receptors in inhibiting LPS-induced TNF-α production in these cells has been previously established (Kreckler et al., 2006). Anti-inflammatory role of A_{2A} receptors has been also shown *in vivo,* as selective A_{2A} agonists attenuate the increase in plasma TNF-α levels induced by LPS in wild-type mice but have no effect in A_{2A}KO animals (Gomez and Sitkovsky, 2003). Our data show that ablation of the A_{2B} receptor gene had no effect on the expression of mRNA encoding A_{2A} receptors in peritoneal macrophages and on NECA-induced suppression of LPS-activated TNF-α release. Although both A_{2A} and A_{2B} receptors can stimulate adenylate cyclase, we found that NECA-induced cAMP accumulation was similar in peritoneal macrophages obtained from wild-type and A_{2B}KO animals, indicating the dominant role of A_{2A} receptors in this process. Of interest, elevation of cAMP in murine macrophages attenuates LPS-induced TNF-α secretion (Mauel et al., 1995), but has no effect on basal IL-6 release (Martin and Dorf, 1991; Tang et al., 1998). Because A_{2B} receptors can couple to other intracellular pathways, e.g. to Gq-phospholipase C pathway not activated by A_{2A} receptors (Feoktistov and Biaggioni, 1995; Ryzhov et al., 2006), it is possible that the differential regulation of TNF-α and IL-6 secretion by A_{2A} and A_{2B} receptors in mouse peritoneal macrophages can be explained by coupling of these receptors to distinct intracellular pathways. Further studies are needed to delineate the signaling pathways linking activation of adenosine receptors to modulation of IL-6 and TNF-α production.

Our study confirmed the previous observation by Yang et al (2006), that A_{2B}KO mice have an increased basal and LPS-stimulated TNF-α release from macrophages, and the higher TNF-α plasma levels. However, our findings are in disagreement with the

interpretation that this reflects activation of A_{2B} receptors by endogenous adenosine suppressing TNF-α release in wild-type mice. Several lines of evidence argue against this possibility. 1) Our experiments in macrophages were conducted in the presence of adenosine deaminase, thus excluding a possibility of receptor activation by endogenous adenosine. Nevertheless, we observed greater basal and LPS-stimulated TNF-α release from A_{2B}KO macrophages, compared to their wild-type control. 2) The A_{2B}KO phenotype was not mimicked by selective A_{2B} antagonists IPDX or MRS 1754 in wild-type mice, since they did not induce an increase in basal or LPS-stimulated TNF-α secretion from peritoneal macrophages; on the contrary, these selective antagonists blocked adenosine-induced IL-6 release. 3) If it is assumed that tonic activation of A_{2B} receptors has anti-inflammatory effects, then a similar pattern should be observed in A_{2A}KO mice, given the overwhelming evidence of the anti-inflammatory role of this receptor subtype and its much higher affinity to adenosine (Fredholm et al., 2001a; Fredholm et al., 2001b). Paradoxically, it has been found that LPS-induced release of TNF-α from peritoneal macrophages (Kreckler et al., 2006) or serum TNF-α levels induced by intraperitoneal LPS challenge *in vivo* (Gomez and Sitkovsky, 2003) were decreased in A_{2A}KO mice. A graphic representation of these findings is shown in Figure 5. It would be erroneous to conclude that A_{2A} receptors are pro-inflammatory simply based on this observation that LPS has less effect on TNF-α release in A_{2A}KO mice. This would disregard the overwhelming evidence of the anti-inflammatory role of this receptor subtype (for review see (Hasko and Cronstein, 2004)). Similarly, we argue that the increase in inflammatory indices observed in A_{2B}KO mice in the absence of stimulation

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of adenosine receptors cannot be interpreted as indicative of putative anti-inflammatory effects of activation of the A_{2B} receptor by adenosine.

Although our experiments argue against an anti-inflammatory effect resulting from activation of A_{2B} receptors by adenosine, they do not exclude the possibility that these receptors are still associated with adenosine-independent regulation of TNF-α production. An alternative and more likely explanation of this phenomenon could be that the A_{2B} receptor protein interacts with other signaling pathways unrelated to adenosine. Recent evidence suggests that adenosine receptors play a role in assembly of multi-protein signaling complexes (Gsandtner et al., 2005; Milojevic et al., 2006; Sun et al., 2006a; Sitaraman et al., 2002; Pacheco et al., 2005). Rearrangement of proteins normally coupled to the A_{2B} receptor as a result of the A_{2B} knockout may affect regulation of TNF-α synthesis or release. Future studies that define the full makeup of A_{2B} receptor complexes with associated signaling components are needed to validate this hypothesis. This phenomenon, however, seems to be irrelevant to potential adenosine-based therapeutic approaches to inflammation. It has been speculated that the pro-inflammatory environment observed in A_{2B}KO mice could be induced with A_{2B} receptor antagonists (Hua et al., 2007). Our current study, however, does not support this hypothesis. It is possible that the phenomenon of enhanced basal and endotoxin-stimulated TNF-α release has functional significance in human cases of A_{2B} mutations or polymorphisms, but this remains speculative.

In conclusion, our study in A_{2B}KO mice has confirmed the concept drawn from previous pharmacological data that stimulation of A_{2B} receptors with adenosine leads to pro-inflammatory events based on the observation that A_{2B} receptors mediate adenosine-

dependent secretion of IL-6, a cytokine generally considered to be pro-inflammatory especially during chronic inflammation (Gabay, 2006). Our data argue against a role of A_{2B} receptors in suppression of LPS-stimulated TNF-α production by adenosine. An increase in basal and LPS-stimulated TNF-α release observed in A_{2B}KO mice is not directly related to adenosine signaling through the A_{2B} receptor, but may represent a loss of other yet unidentified functions of this protein, or reflect a developmental adaptation to the A_{2B} receptor gene ablation.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Expression of adenosine receptors in wild-type (WT) and A_{2B}KO mouse peritoneal macrophages.

Real-time RT-PCR analysis of mRNA encoding adenosine receptor subtypes was performed as described in the Methods section. Values are expressed as means±SEM of three separate cell preparations. ND, no transcripts detected

Figure 2. Intracellular cAMP levels in wild-type (WT) and A_{2B}KO mouse peritoneal macrophages.

Cyclic AMP accumulation was measured in cells incubated in the presence of 10 μM NECA, 1 μM forskolin or their vehicle (Basal) as described in the Methods section. Values are expressed as means±SEM of four separate cell preparations.

Figure 3. Effect of A_{2B} receptor gene ablation on IL-6 plasma levels and secretion from peritoneal macrophages.

A, Mouse peritoneal macrophages isolated from wild type (WT) and A_{2B}KO mice were incubated in the presence of 10 μM NECA or its vehicle (Basal) for 16 h. IL-6 was measured in culture media and expressed as pg/mg of total cell protein. Data are means±SEM of five separate cell preparations. Asterisks indicate statistical difference from basal (***$p<0.001$, two-way ANOVA with Bonferroni's post-test). B, Wild-type (WT) and A_{2B}KO mice received i.p. injections of 0.5 mg/kg NECA or its vehicle (Basal). Plasma IL-6 concentrations were analyzed 3 h later. Data are presented as means±SEM

of four animals per group. Asterisks indicate statistical difference from basal values (***p<0.001, two-way ANOVA with Bonferroni's post-test).

Figure 4. Effect of A_{2B} receptor gene ablation on TNF-α plasma levels and secretion from peritoneal macrophages.

A, Mouse peritoneal macrophages isolated from wild-type (WT) and A_{2B}KO mice were incubated in the presence of 10 μM NECA or its vehicle (Basal) for 16 h. TNF-α was measured in culture media and expressed as pg/mg of total cell protein. Data are presented as means±SEM of five separate cell preparations. B, Macrophages stimulated with 10 ng/ml LPS were incubated in the presence of 10 μM NECA (LPS+NECA) or its vehicle (LPS) for 16 hours. TNF-α was measured in culture media and expressed as pg/mg of total cell protein. Data are presented as means±SEM of four separate cell preparations. Asterisks indicate statistical difference from LPS values (*p<0.05, **p<0.01, two-way ANOVA with Bonferroni's post-test). C, Wild-type (WT) and A_{2B}KO mice received i.p. injections of 0.5 mg/kg NECA (LPS+NECA) or its vehicle (LPS) 10 min prior to i.p. injections of 10 mg/kg LPS. Plasma TNF-α concentrations were analyzed 1.5 h later. Data are presented as means±SEM of five animals per group. Asterisks indicate statistical difference from LPS values (***p<0.01, two-way ANOVA with Bonferroni's post-test).

Figure 5. Comparison of previously published results on the effect of gene ablation of A_{2A} and A_{2B} receptors on LPS-induced TNF-α release.

A, Genetic ablation of A_{2A} receptors was associated with attenuated TNF-α secretion induced by LPS in peritoneal macrophages *ex vivo* (left axis), and with attenuated TNF-α levels induced by intraperitoneal administration of LPS *in vivo* (right axis). Data derived from previously published results (Kreckler et al., 2006; Gomez and Sitkovsky, 2003) are shown here for comparison purposes only. B, Genetic ablation of A_{2B} receptors was associated with augmented TNF-α secretion induced by LPS in peritoneal macrophages *ex vivo* (left axis), and with augmented plasma TNF-α levels induced by intraperitoneal administration of LPS *in vivo* (right axis). Data derived from previously published results (Yang et al., 2006) are shown here for comparison purposes only.

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Table 1. Effect of selective A_{2B} antagonists on IL-6 release (pg/mg) from peritoneal macrophages.

The selective A_{2B} antagonists IPDX (10 μM), MRS 1754 (1 μM) or their vehicle were added to cells, which were then incubated in the absence (Basal) or presence of 10 μM NECA for 16 hours. Data are means±SEM of five separate cell preparations. Asterisks indicates significant difference from vehicle (** $p<0.01$, one-way ANOVA with Dunnett's post-test).

	Vehicle	IPDX	MRS 1754
Basal	64.2±4.86	62.3±11.4	60.3±13.4
NECA	371.8± 22.9	105.8± 19.4**	103.9± 16.8**

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Table 2. Effect of selective A_{2B} antagonists on TNF-α release (pg/mg) from peritoneal macrophages.

The selective A_{2B} antagonists IPDX (10 μM), MRS 1754 (1 μM) or their vehicle were added to cells, which were then incubated in the absence (Basal, LPS) or presence of 10 μM NECA (NECA, LPS+NECA) and in the absence (Basal, NECA) or presence of 10 ng/ml LPS (LPS, LPS+NECA) for 16 hours. Data are means±SEM of five separate cell preparations. No significant difference from vehicle was found by one-way ANOVA with Dunnett's post-test.

	Vehicle	IPDX	MRS 1754
Basal	45.6±6.5	44.6±7.3	43.6±5.4
NECA	34.6±7.2	39.9±6.4	38.2±9.4
LPS	4640±903	3459±599	3461±643
LPS+NECA	2012±430	1571±191	1493±336

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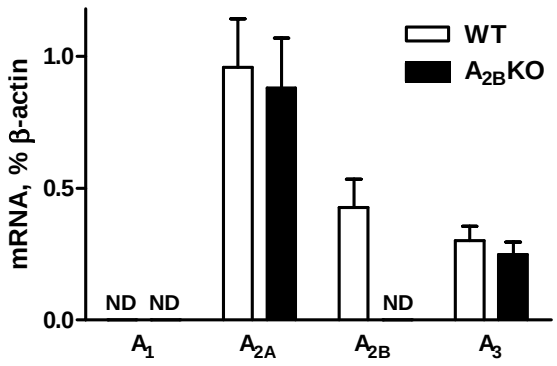

Figure 1

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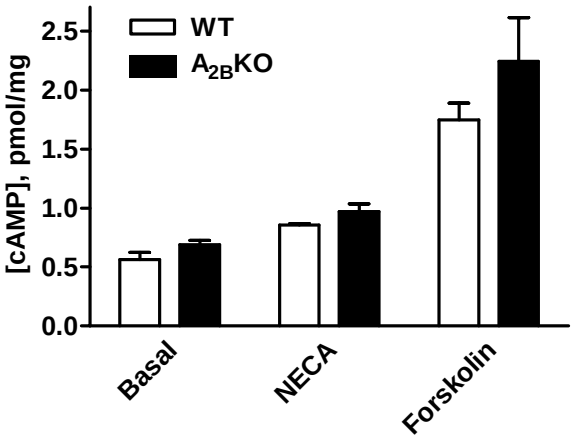

Figure 2

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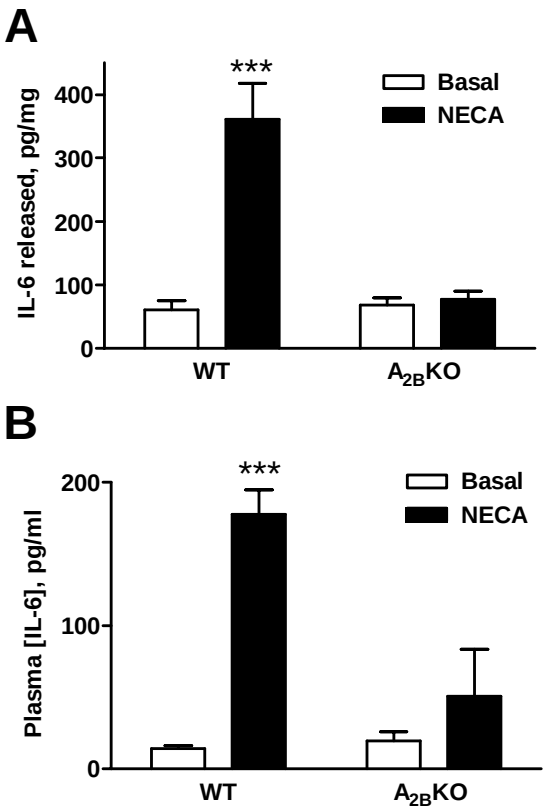

Figure 3

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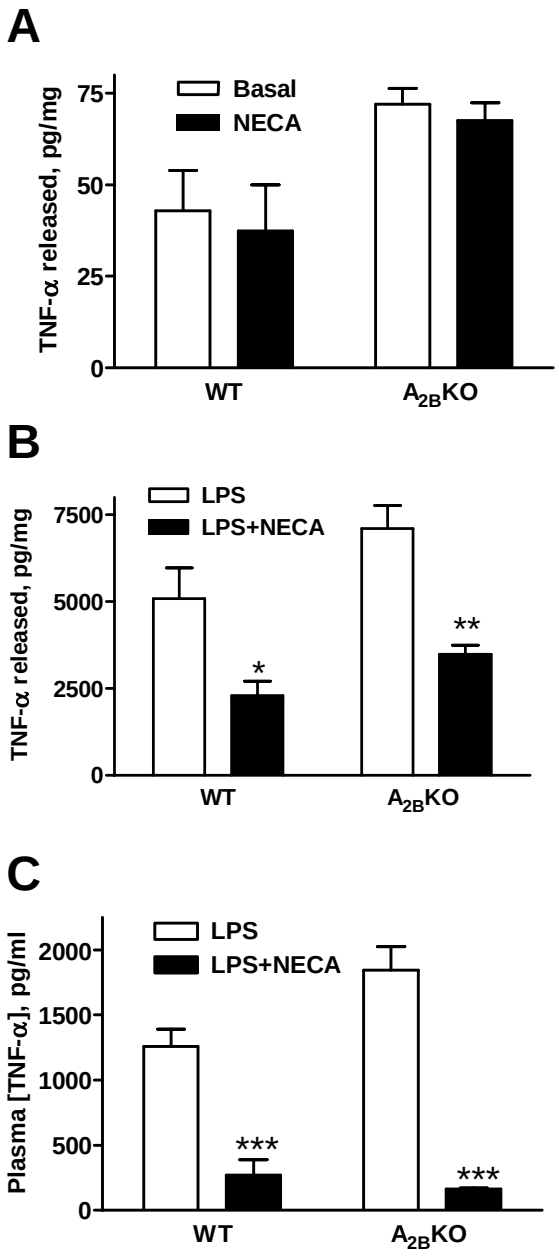

Figure 4

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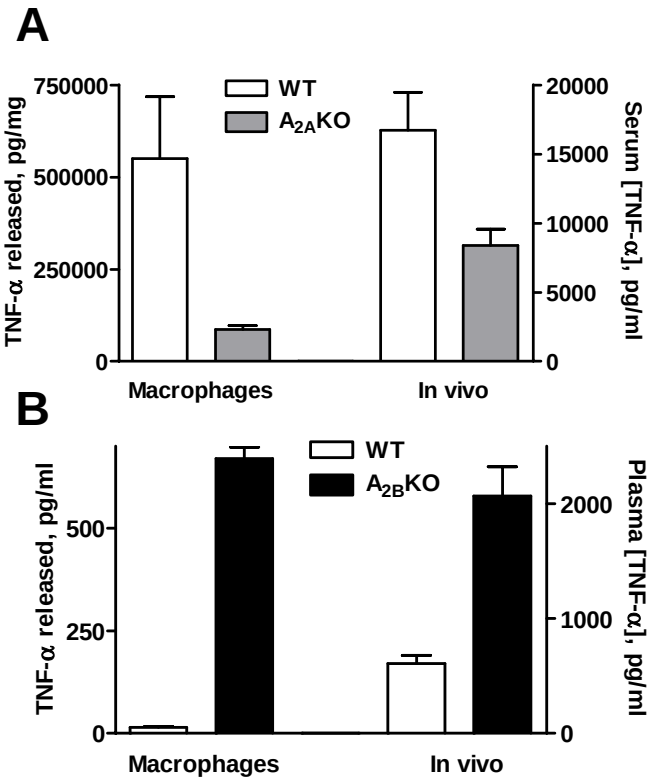

Figure 5