Lung Injury

Acute Receptor DAMPENS ENDOTOXIN-INDUCED ACUTE LUNG INJURY

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Signaling through the A2B Adenosine Receptor Dampens Endotoxin-Induced Acute Lung Injury

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Sepsis and septic acute lung injury are among the leading causes for morbidity and mortality of critical illness. Extracellular adenosine is a signaling molecule implicated in the cellular adaptation to hypoxia, ischemia, or inflammation. Therefore, we pursued the role of the A2B adenosine receptor (AR) as potential therapeutic target in endotoxin-induced acute lung injury. We gained initial insight from in vitro studies of cultured endothelia or epithelia exposed to inflammatory mediators showing time-dependent induction of the A2BAR (up to 12.9 ± 3.4-fold, p < 0.05). Similarly, murine studies of endotoxin-induced lung injury identified an almost 4.6-fold induction of A2BAR transcript and corresponding protein induction with LPS exposure. Studies utilizing A2BAR promoter constructs and RNA protection assays indicated that A2BAR induction involved mRNA stability. Functional studies of LPS-induced lung injury revealed that pharmacological inhibition or genetic deletion of the A2BAR was associated with dramatic increases in lung inflammation and histologic tissue injury. Studies of A2BAR bone marrow chimeric mice suggested pulmonary A2BAR signaling in lung protection. Finally, studies with a specific A2BAR agonist (BAY 60-6583) demonstrated attenuation of lung inflammation and pulmonary edema in wild-type but not in gene-targeted mice for the A2BAR. These studies suggest the A2BAR as potential therapeutic target in the treatment of endotoxin-induced forms of acute lung injury. The Journal of Immunology, 2010, 184: 000–000.

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Abbreviations used in this paper: ADA, adenosine deaminase; ALI, acute lung injury; AR, adenosine receptor; DBR, 5,6-dichlorobenzimidazole; HIF, hypoxia-inducible factor; HMEC, human microvascular endothelial cell; MPO, myeloperoxidase; PMN, polymorphonuclear neutrophil; WT, wild-type.

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Material and Methods
Cell culture and inflammatory stimulation
Human microvascular endothelial cells (HMEC-1) and cultured pulmonary epithelial cells (A549 cells; LGC Standards, WeSEL, Germany) were cul- tured as described previously (17–20). Primary pulmonary endothelial cells (HMEVEC-L; Lonza Walkersville, Walkersville, MD) were cultured under the supplier’s instructions. Cells were grown to full confluency and stim- ulated with 10 ng/ml LPS (Sigma-Aldrich, Taufkirchen, Germany), 10 ng/ml IL-1β (PromoKine, Heidelberg, Germany), 70 ng/ml IL-4 (PromoKine), and 20 ng/ml IL-6 (PromoKine) for 6, 12, and 24 h.

Murine LPS inhalation model
Experiment protocols were approved by the University of Tübingen, Tübingen, Germany, or the University of Colorado at Denver, Denver, CO. They were also in accordance with the German Law on the Protection of Animals and the National Institutes of Health (Bethesda, MD) guidelines for use of live animals. C57BL/6J mice (Charles River Laboratories, Wilmington, MA), A2BAR+/- mice on a C57BL/6J background, or age-, gender-, and weight-matched littermate controls were bred and genotyped as described previously (12, 21). Mice at age 8–12 wk were exposed to aerosolized LPS in a cylindrical chamber connected to an air nebulizer (MicroAir; Omron Healthcare, Mannheim, Germany). The outlet of the chamber was to a vacuum pump producing a constant flow rate. LPS from Escherichia coli B026 (Sigma-Aldrich) was dissolved in 0.9% saline (500 μg/ml). A2BAR antagonist MRS1754 (Tocris, Bristol, U.K.) was dissolved in 0.9% saline (2.4 μg/ml), and mice were allowed to inhale for 30 min prior to LPS exposure. Control mice were exposed to saline aerosol. In other studies, mice were pretreated with the A2BAR agonist BAY 60-5683 (2 mg/kg i.p.; Bayer HealthCare AG, Wuppertal, Germany) or vehicle (21). Mice were sacrificed 4 h after LPS exposure; the remaining blood was removed from the pulmonary circulation by injecting 1 ml PBS into the right ventricle. Lungs were excised and immediately frozen at −80°C for further analysis.

Human and mouse transcriptional analysis
Total RNA was extracted as described previously (12, 21–28) using the RNA isolation kit Nucleospin RNA II (Machery & Nagel, Düren, Germany). RNA was washed, and the concentration was quantified. cDNA synthesis was performed by using the iScript cDNA Synthesis Kit (Bio- Rad, Munich, Germany) according to the manufacturer’s instructions. Quantitative real-time RT-PCR (iCycler; Bio-Rad) was performed to examine A2BAR expression levels. qPCR Master Mix contained 1 μM sense and 1 μM antisense primers with iQ SYBR Green (Bio-Rad). To quantify the change in A2BAR transcript levels, the following primer sets were used: sense, 5’-ATC TCC AGG TAT CTT CTC-3’; antisense, 5’-GTT GGC ATA ATC ACC ACA GCA-3’. Samples were controlled for β-actin expression using following primers: sense, 5’-GGA GAA AAT CTG CCA CCA CA-3’; antisense, 5’-AGA GGC GTA CAG GGA TAG CA-3’. To analyze murine A2BAR transcript, lung tissue was excised and total RNA was isolated. A2BAR mRNA levels were quantified using sense primer 5’-GGG CAG CAA CTC AGA AAA CT-3’ and antisense primer 5’-AGA GG ACT TCG TCT CTC CA-3’. Murine β-actin expression was evaluated with sense primer 5’-GCC TTG GCA CAG CAT CAC CAT GAA GA-3’ and antisense primer 5’-TCT GCT GGA AGG TGG ACA G-3’, murine IL-1β expression was evaluated with sense primer 5’-GCC TCT TAG CAC CAT CAC TCA TT-3’ and antisense primer 5’-CAC ACC AGG AGG TTA TCA TC-3’, murine IL-6 expression was evaluated with sense primer 5’-ACC GAT CCT AAC AGG GCG TCG TCT CTC-3’ and antisense primer 5’-CAC CCT AGT GCT CAG GGC TCG TCT CTC-3’. Samples were treated with the transcriptional inhibitor 5,6-dichlorobenzimidazole (DRB; 50 μM) to prevent de novo transcription of mRNA. RNA was harvested at 0, 1, 2, 3, and 4 h posttreatment with DRB using TRIzol. The degradation rate of A2BAR mRNA was evaluated by real-time RT-PCR (n = 3) and calculated relative to the mRNA levels at 0 h.

ELISA for IL-6 from lung tissue
The snap-frozen lungs were thawed, weighed, and transferred to different tubes on ice containing 1 ml Tissue Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL). The lung tissues were homogenized at 4°C. Lung homogenates were centrifuged at 9000 × g for 10 min at 4°C. Supernatants were transferred to clean microcentrifuge tubes, frozen on dry ice, and thawed on ice. Total protein concentrations in the lung tissue homogenates were determined using a bicinchoninic acid kit (Pierce Biotechnology). Lung tissue homogenates were diluted with 50% assay diluent and 50% Tissue Protein Extraction Reagent to a final protein concentration of 400 μg/ml. IL-6 levels were evaluated in lung tissue homogenates using a mouse ELISA kit (R&D Systems), in accordance with the manufacturer’s instructions.

Human and mouse protein analysis
Cell culture and mouse tissue samples were normalized for protein levels before applying them to nonreducing conditions to SDS-containing polyacrylamide gels. Abs used for Western blotting included rabbit polyclonal anti-A2BAR (Santa Cruz Biotechnology, Santa Cruz, CA) for human and murine A2BAR analysis. Goat polyclonal anti-IL-6 (Santa Cruz Biotechnology) and goat polyclonal anti-TNF-α (Santa Cruz Biotechnology) were used to analyze murine protein levels. β-Actin was stained using rabbit anti-β-actin Ab (Cell Signaling Technology, Danvers, MA). Blots were washed, and species-matched alkaline phosphatase-conjugated secondary Abs were added: goat anti-rabbit IgG (Santa Cruz Biotechnology) and donkey anti-goat IgG (Santa Cruz Biotechnology). Labeled bands from stripped blots were developed using a detection buffer containing 5-bromo-4-chloro-3-indolyl phosphate (AppliChem, Darmstadt, Germany) and NBT (AppliChem).

Histopathological evaluation of endotoxin-induced ALI
Following LPS inhalation, mice were euthanized and lungs were fixed by instillation of 10% formaldehyde solution through a tracheal cannula. Lungs were then embedded in paraffin and stained with H&E. Three random tissue sections from four different lungs in each group were examined by a pathologist who was blinded to the genetic background/treatment of the mice. Lung injury was scored according to the following criteria: 1) alveolar congestion, 2) hemorrhage, 3) infiltration or aggregation of neutrophils in airspace or vessel wall, and 4) thickness of the alveolar wall/hyaline membrane formation. For each subject, a five-point scale was applied: 0, minimal (little) damage; 1+, mild damage; 2+, moderate damage; 3+, severe damage; and 4+, maximal damage. Points were added up and are expressed as median ± range of injury score.

Immunofluorescent staining
After animals were killed, lungs were embedded in paraffin and sectioned. Tissue sections were placed on slides, air-dried, and fixed in methanol and subsequently in 4% acetone. Air-dried tissue sections were washed three times in PBS after each step of staining and blocked with 5% nonfat milk for 20 min. Samples were incubated for 60 min with the following Abs: polyclonal rabbit anti-A2BAR Ab (Santa Cruz Biotechnology) at a dilution of 1:1000 as primary Ab and rabbit IgG fraction (Dako/Cytomation, Glostrup, Denmark) as negative control. Alexa Fluor 488 goat anti-rabbit (Invitrogen, Carlsbad, CA) was used as secondary Ab. Then the slides were covered with DAPI (Molecular Probes, Eugene, OR) to perform nuclear counterstaining.

Quantification of pulmonary neutrophils and pulmonary edema
Pulmonary infiltration by polymorphonuclear neutrophils (PMNs) was quantified by enzymatic assay for the azurophilic neutrophil granule protein myeloperoxidase (MPO). Whole sections of murine lung were snap-frozen at harvest, then thawed and homogenized in PBS/1% Triton X-100 (Sigma-Aldrich). Samples were acidified in PBS/citrate buffer, diluted 1:1 with ABTS. Resulting supernatant was measured at 405 nm. To quantify pulmonary edema, wet-to-dry ratios were measured as
described previously (30). In short, following LPS inhalation lungs were excised en bloc. The weight of tissue samples was obtained immediately to prevent evaporative fluid loss of the tissues. Lungs were then lyophilized for 48 h, and the dry weight was measured. Wet-to-dry ratios were then calculated as milligrams of water per milligram of dry tissue.

**Generation of bone marrow chimeras**

To define the contribution of the myeloid and lung tissue-specific A2BAR bone marrow, chimeric mice were generated in which bone marrow was ablated by radiation in wild-type (WT) mice (C57BL/6J) followed by reconstitution with bone marrow derived from previously characterized mice gene-targeted for A2BAR\(^{2/2}\) and vice versa. Transplantation efficiencies and details of the protocol were described previously (11, 12, 15, 16). To control for nonspecific radiation effects, bone marrow was transplanted from WT \(\rightarrow\) WT and A2BAR\(^{2/2}\) \(\rightarrow\) A2BAR\(^{2/2}\) mice. Male donor mice (8- to 10-wk-old, 20–25 g) were euthanized, marrow was harvested by flushing the marrow cavity, and bone marrow cells were then centrifuged at 400 \(\times\) g for 5 min, resuspended, and counted. Recipient mice (8–10 wk old, 20–25 g) were irradiated with a total dose of 12 Gy from a \(^{137}\)Cs source. Immediately after irradiation, \(1 \times 10^7\) bone marrow cells were injected in 0.3 ml 0.9% NaCl into the jugular vein of each recipient. The resulting chimeric mice were housed in microisolators for at least 8 wk before experimentation and were fed with water containing tetracycline (100 mg/l) during the first two weeks following bone marrow transplantation. Preliminary experiments using the same conditioning regimen and transplanting CD45.1+ bone marrow into irradiated CD45.1+ mice resulted in \(95\%\) chimerism in B cells, neutrophils, and monocytic cells and \(\sim 85\%\) chimerism in CD4+ and Ly6G+ T cells of recipient mice (11, 12, 15, 16). After successful transplantation, mice were again exposed to LPS inhalation and sacrificed, and lung damage was evaluated as described above.

**Statistical analysis**

Data are presented as mean \(\pm\) SD from four to six animals per condition. We performed statistical analysis using the Student \(t\) test (two-tailed, \(p < 0.05\)). Lung injury score was analyzed with the Kruskal-Wallis rank test. A value of \(p < 0.05\) was considered statistically significant.

**Results**

**In vitro exposure to inflammatory stimuli increases A2BAR transcript and protein levels**

In order to investigate the role of the A2BAR in endotoxin-induced ALI, we first pursued in vitro studies of A2BAR expression following exposure with different inflammatory stimuli. These studies are based on previous work indicating an important contribution of the A2BAR in dampening inflammation caused by hypoxia (16) and other reports demonstrating transcriptionally regulated pathways for the A2BAR (e.g., involving the transcription factor hypoxia-inducible factor [HIF]-1\(\alpha\)) (11, 18, 20, 22, 29). On the basis of these studies, we pursued the hypothesis that A2BAR expression is enhanced following exposure to inflammatory stimuli. Previous studies have indicated that pulmonary A2BAR activity includes relevant expression on pulmonary epithelial cells (10) or vascular endothelia (12–14). As such, we modeled this event by exposing cultured pulmonary epithelial cells (A549) or vascular endothelial cells (HMEC-1) to a panel of inflammatory mediators, including PGE2, IL-1\(\beta\), IL-4, and IL-6 over a time-
course of up to 24 h and assessed regulation of A2ABR expression by real-time RT-PCR (Fig. 1A–D). In fact, these studies revealed dramatic increases in A2ABR transcript levels with exposure to inflammatory stimuli. To gain insight into the mechanisms of A2BAR induction, we profiled the influence of the above inflammatory mediators on previously characterized A2BAR luciferase reporter constructs (29). These studies revealed no significant changes in promoter activity under any of the conditions tested (data not shown). On the basis of these findings, we investigated whether A2BAR mRNA is protected against degradation in the absence of de novo transcription (i.e., change in mRNA half-life). On the basis of the prominent upregulation of A2BAR transcript in response to PGE2 stimulation (Fig. 1A), we investigated the potential for PGE2 to stabilize A2BAR mRNA. In order to ascertain the effects of PGE2 on the enhancement of A2BAR levels in the absence of de novo synthesis, we evaluated the ability for PGE2 to posttranscriptionally regulate A2BAR levels in HMEC cells. Accordingly, we treated HMECs with 0.5 μM PGE2, followed by a 4 h treatment with 50 μM DRB. Using real-time RT-PCR analysis, we found that PGE2 treatment is associated with increased A2BAR mRNA stability (p < 0.05, n = 3). These findings implicate that PGE2 treatment elicits anti-inflammatory signaling pathways involving enhanced mRNA stability of the A2BAR.

Further to our findings in established endothelial cultures of nonpulmonary origin, we sought to determine whether this regulation occurred in a primary pulmonary cell line. Accordingly, we exposed primary pulmonary endothelial cells (HMVEC-L) to IL-6. Consistent with the previous studies of immortalized cell lines, we found time-dependent increases in A2BAR transcript levels in primary pulmonary endothelial cells (Fig. 1F). Similarly, A2BAR protein levels were elevated in a dose-dependent fashion following exposure to IL-6 over 24 h (Fig. 1G). Taken together, these data indicate increased A2BAR transcript and protein levels following inflammatory stimulation of cultured pulmonary epithelial cells, vascular endothelia, or primary pulmonary endothelial cells—at least in part through posttranscriptional regulation of A2BAR mRNA.

The A2BAR is induced during septic lung injury in vivo

After having shown that inflammatory stimulation of pulmonary cells (endothelia and epithelia) is associated with robust induction of the A2BAR, we next pursued these findings in an in vivo model of endotoxin-induced ALI. For this purpose, we used LPS inhalation. We exposed C57BL/6J mice over 30 min to inhaled LPS in a model system that we had used previously in studies on the role of CD39- and CD73-dependent adenosine generation in endotoxin-induced ALI (5). Control animals underwent similar

**FIGURE 2.** A2BAR expression following LPS inhalation in vivo. Mice were exposed to 30 min of LPS inhalation, and animals were sacrificed after 4 h. A., Pulmonary A2BAR transcript levels were assessed by real-time RT-PCR. Data were calculated relative to the internal housekeeping gene (β-actin) and are expressed as mean fold change compared with control (−LPS) ± SD (n = 9). B., Western blot analysis of A2BAR protein following in vivo exposure to inhaled LPS. The same blot was stripped and reprobed for murine β-actin to control for loading conditions. One representative of four Western blots is displayed. C, Pulmonary immunohistochemistry for the A2BAR following LPS exposure. Lungs from mice exposed to 30 min of LPS inhalation or vehicle control were harvested. Sections were stained with Abs specific for murine A2BAR (green) or isotype controls. DAPI was used for nuclear counterstain (blue) (original magnification ×400). One representative image from three pulmonic sections is displayed.
Pharmacological inhibition of A2BAR signaling is associated with enhanced lung inflammation following LPS inhalation

To study a functional role of the A2BAR in septic lung injury, we first pursued pharmacological studies. Here, we used MRS1754, a compound demonstrated to act as a specific antagonist of the A2BAR in vivo (8). On the basis of other studies showing high expression levels of the A2BAR in pulmonary epithelial cells, we decided to employ an inhaled route of administration. For this purpose, MRS1754 (2.4 μg/ml) was given via nebulizer over 30 min, followed by 30 min of LPS inhalation. Lungs were excised after 4 h, and inflammatory markers were determined. LPS treatment was associated with increases in pulmonary transcript levels of IL-1β (Fig. 3A), IL-6 (Fig. 3B), and TNF-α (Fig. 3C) in vehicle-treated animals. However, inhibition of the A2BAR with MRS1754 dramatically augmented the effects of LPS administration (Fig. 3A–C). Similarly, pulmonary IL-6 protein levels were elevated in conjunction with LPS treatment and showed additional increases following A2BAR inhibition (Fig. 3D). Taken together these data indicate that pretreatment with a pharmacological inhibitor of the A2BAR synergistically enhances endotoxin-induced increases in lung inflammation during ALI.

FIGURE 3. Influence of inhaled A2BAR antagonist MRS1754 on lung inflammation following LPS inhalation in vivo. Eight- to 12-wk-old C57BL/6J mice matched in age, gender, and weight were pre-exposed to 30 min of inhaled A2BAR antagonist (MRS1754) or vehicle, followed by 30 min of LPS or vehicle inhalation. Animals were sacrificed after 30 min, and pulmonary transcript levels of (A) IL-1β, (B) IL-6, or (C) TNF-α were assessed by real-time RT-PCR. Data were calculated relative to the internal housekeeping gene (β-actin) and are expressed as mean fold change compared with control (−LPS, −MRS1754) ± SD (n = 4–7). D, Western blot analysis of IL-6 protein. The same blot was stripped and reprobed for murine β-actin to control for loading conditions. One representative of three Western blots is displayed. Note enhanced lung inflammation following A2BAR antagonist treatment following LPS exposure.
of pulmonary versus myeloid A2BAR signaling effects. Previous studies had demonstrated functional A2BAR expression on inflammatory cells (e.g., neutrophils or macrophages) (14, 16) and on pulmonary tissues (e.g., pulmonary epithelia and vascular endothelia) (10, 11, 18, 31). Therefore, we generated A2BAR bone marrow chimeric mice—as we have done previously (11, 12)—to study the contribution of pulmonary versus myeloid A2BARs in bone marrow chimeric mice—as we have done previously (11, 12)—to pulmonary tissues (e.g., pulmonary epithelia and vascular endothelial cells (e.g., neutrophils or macrophages) (14, 16) and on endotoxin-induced ALI. As expected, A2BAR bone marrow chimeric mice showed a phenotype similar to that in WT mice (21). Therefore, we pretreated mice with BAY 60-6583 30 min prior to LPS exposure (2 mg/kg i.p.). Pretreatment with BAY 60-6583 was associated with attenuated pulmonary myeloperoxidase elevations following LPS treatment (Fig. 7A) indicating diminished LPS-elicited inflammatory cell accumulation. Similarly, LPS-elicited increases in pulmonary IL-6 levels (Fig. 7B) or LPS-dependent increases in lung water (Fig. 7C) were significantly attenuated. In contrast, A2BAR treatment was ineffective in abrogating these inflammatory parameters in A2BAR−/− mice (Fig. 7D, 7E), confirming the specificity and efficacy of BAY 60-6583 for the A2BAR. Taken together, these studies identify the A2BAR as a pharmacological target for endotoxin-induced ALI.

A2BAR agonist treatment in endotoxin-induced ALI

After having shown that pharmacological inhibition or genetic deletion of the A2BAR is associated with a more severe degree of lung inflammation and pulmonary edema, we next pursued the hypothesis that A2BAR agonist treatment will attenuate LPS-induced lung injury. For this purpose, we used a recently described non-adenosine-like agonist of the A2BAR (BAY 60-6583). As such, previous studies have provided genetic in vivo evidence for A2BAR activity and specificity by showing significant reduction of myocardial infarct size in WT mice but not in A2BAR−/− mice (21). Therefore, we pretreated mice with BAY 60-6583 30 min prior to LPS exposure (2 mg/kg i.p.). Pretreatment with BAY 60-6583 was associated with attenuated pulmonary myeloperoxidase elevations following LPS treatment (Fig. 7A) indicating diminished LPS-elicited inflammatory cell accumulation. Similarly, LPS-elicited increases in pulmonary IL-6 levels (Fig. 7B) or LPS-dependent increases in lung water (Fig. 7C) were significantly attenuated. In contrast, A2BAR treatment was ineffective in abrogating these inflammatory parameters in A2BAR−/− mice (Fig. 7D, 7E), confirming the specificity and efficacy of BAY 60-6583 for the A2BAR. Taken together, these studies identify the A2BAR as a pharmacological target for endotoxin-induced ALI.

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Discussion

Over 40% of patients with sepsis go on to develop ALI, which is the most common cause of death among death in these patients (3). At present, research studies to define novel therapeutic approaches for endotoxin-induced ALI is an area of intense investigation. On the basis of previous studies showing a potential therapeutic role for signaling events through the A2BAR in attenuating mucosal inflammation (16, 24, 32), we pursued the hypothesis that the A2BAR represents a therapeutic target during LPS-induced lung injury. Indeed, bacterial toxins, such as LPS, are a common cause of lung injury in patients suffering from sepsis (33). In the studies presented here, we demonstrated induction of the A2BAR following exposure to inflammatory stimuli in cultured pulmonary epithelia or vascular endothelia in vitro or in an in vivo model investigating the lungs of mice exposed to LPS inhalation. Animals were sacrificed after 4 h and (A) pulmonary transcript levels of IL-6 were determined by real-time RT-PCR. B, Lung water content. Data were calculated relative to the internal housekeeping gene (β-actin) and are expressed as mean fold change compared with control (WT → WT treated with vehicle) ± SD (n = 4–7).

Finally, pretreatment with A2BAR agonist (BAY 60-6583) significantly attenuated lung inflammation and pulmonary edema in WT animals but was ineffective in A2BAR−/− mice. Taken together, such studies indicate a potential role for A2BAR signaling in dampening lung inflammation and pulmonary edema during LPS-induced lung injury.

It has been previously shown that A2BAR expression is upregulated in response to proinflammatory cytokines, such as TNF-α. In contrast to these findings, the there are few studies to date that show the mechanism of how A2BR protein expression is regulated in response to inflammatory stimuli. As such, previous studies had identified transcriptionally regulated alterations of A2BAR expression during hypoxia-elicited inflammation. These studies demonstrated a selective induction of the A2BAR following exposure to ambient hypoxia. In contrast, transcript levels of other ARs were either repressed or unaltered (18). Subsequent studies identified a previously unrecognized binding site for HIF-1 within the promoter region of the A2BAR (29). Additional studies investigating the promoter activity, functional chromatin binding, and HIF loss-of-function demonstrated a critical role of HIF-1α in mediating hypoxia-associated induction of the A2BAR (29). Other studies demonstrated HIF-dependent induction of the A2BAR during myocardial ischemia (21, 22). Similarly, a recent study indentified a transcriptionally regulated pathway elicited by hypoxia involving HIF-2α–dependent induction of the A2AAR (34).
Although these studies demonstrate transcriptionally regulated alterations of AR gene expression, the present studies could not find alterations of A2BAR promoter activity elicited by inflammatory mediators. In contrast, the present studies indicate that increases in A2BAR following exposure to inflammatory stimuli involve alterations in mRNA stability. Further studies are however required to elucidate the signaling mechanisms underpinning the stabilization of A2BAR stabilization.

Similar to the present results, other studies confirmed a role of adenosine generation and signaling in different forms of inflammatory diseases. For example, genetic deletion of CD39 or CD73—the key enzymes in extracellular adenosine generation from precursor molecules (8, 18)—results in increased lung inflammation and pulmonary edema when exposed to ventilator-induced lung injury (30). Similarly, cd39−/− or cd73−/− mice demonstrate signs of increased neutrophil trafficking into the lungs upon LPS exposure. As such, pulmonary CD39 and CD73 transcript levels were elevated following LPS exposure in vivo. Moreover, LPS-induced accumulation of PMNs into the lungs was enhanced in cd39−/− or cd73−/− mice, particularly into the interstitial and intra-alveolar compartment. Such increases in PMN trafficking were accompanied by corresponding changes in alveolar-capillary leakage. Similarly, inhibition of extracellular nucleotide phosphohydrolysis with the nonspecific ecto-nucleoside triphosphate diphosphohydrolases inhibitor POM-1 confirmed increased pulmonary PMN accumulation in WT mice but not in gene-targeted mice for cd39 or cd73. Finally, treatment with apyrase or nucleotidase was associated with attenuated pulmonary neutrophil accumulation and pulmonary edema during LPS-induced lung injury (5). Together, such data indicate the likelihood that CD39- and CD73-dependent adenosine production protects from LPS- or ventilator-induced lung injury (5, 30).

Previous research work had identified different ARs in lung protection. Specifically, several studies have pointed toward an important role of A2AAR signaling. Indeed, it has been demonstrated that A2AAR−/− mice exhibit a more severe phenotype when exposed to different models of inflammation or sepsis (35–37). Similarly, studies of LPS-induced lung injury revealed a contribution of myeloid A2AAR signaling in lung protection (38). Utilizing studies with bone marrow chimeric mice in conjunction with studies of myeloid-specific A2AAR deletion, the authors found a critical role of myeloid A2AAR signaling in attenuating PMN trafficking into the lungs. Furthermore, an important role of pulmonary A2BAR signaling in lung protection during mechanical ventilation-induced injury has recently been demonstrated (15). In conjunction with the findings from the present studies, it appears that LPS-induced lung injury could be attenuated by extracellular adenosine signaling events involving A2AARs expressed predominantly on inflammatory cells and A2BARs expressed predominantly on pulmonary tissues.

In conjunction with the present studies, several other studies indicated the A2BAR in disease models that frequently occur in patients suffering from sepsis. As such, the A2BAR agonist BAY 60-6583 has been implicated in the treatment of intestinal ischemia induced by intermittent ligation of intestinal blood flow, followed by reperfusion (24). Similarly, an anti-inflammatory and tissue protective effect of A2BAR signaling had been observed in models of acute intestinal inflammation (32). Moreover, activation of the A2BAR has been shown to decrease vascular leakage in the setting of hypoxia-induced vascular leakage (11) or acute kidney injury (12). It is important to point out that the relatively selective role of A2BAR signaling in these models may be related to the robust induction of these A2BARs under these conditions. Although A2BAR−/− mice appear phenotypically normal and do not exhibit signs of immunologic defects when housed in a pathogen-free environment (21), the A2BAR appears to play an important role under disease conditions associated with its induction (11, 15, 18, 21, 22). Moreover, a coordinated response of increased adenosine production (18), attenuated adenosine uptake (39, 40), and decreased intracellular adenosine metabolism (41) may further contribute to the elevation of extracellular adenosine levels, resulting in sufficient adenosine concentrations capable of activating the relatively “adenosine-insensitive” A2BAR. Moreover, recent studies indicate that the neuronal guidance molecule netrin-1 is induced during conditions of inflammatory hypoxia and may contribute to enhanced extracellular signaling events through the A2BAR (16). Taken together, such studies highlight a potential role for the A2BAR as a therapeutic target during sepsis.

In contrast to the beneficial effects of increased adenosine production and signaling during ALL, there is some evidence suggesting a potentially detrimental role of chronically elevated adenosine levels (42–45). For example, levels of adenosine are increased in the lungs of asthma patients (46) and correlate with the degree of inflammatory insult (47). At present, it is not entirely clear whether such elevations of adenosine are part of a protective pathway to dampen lung inflammation or play a provocative role of adenosine in asthma or chronic obstructive pulmonary disease (48). For example, mice incapable of extracellular adenosine generation (cd73−/− mice) exhibit a more severe phenotype in bleomycin-induced lung injury, indicating a protective role of extracellular adenosine signaling in this chronic model of lung disease (49). In contrast, adenosine deaminase (ADA)-deficient mice develop signs of chronic lung inflammation in association with dramatically elevated pulmonary adenosine levels. In fact, ADA-deficient mice die within weeks after birth from severe respiratory distress (50), and pharmacological studies suggest that attenuation of adenosine signaling through the A2BAR may reverse the severe pulmonary phenotypes in ADA-deficient mice (44, 50). To address these findings on a genetic level, a very elegant study examined the contribution of A2BAR signaling in this model via a genetic approach by generating ADA/A2BAR double knockout (KO) mice (51). The authors’ initial hypothesis was that genetic removal of the A2BAR from ADA-deficient mice would lead to diminished pulmonary inflammation and damage. Unexpectedly, ADA/A2BAR double KO mice exhibited enhanced pulmonary inflammation and airway destruction. Marked loss of pulmonary barrier function and excessive airway neutrophilia are thought to contribute to the enhanced tissue damage observed. These findings support an important protective role for A2BAR signaling during acute stages of lung disease (51).

Taken together, the present studies indicate a protective role of A2BAR signaling in endotoxin-driven lung injury and suggest a potential role for A2BAR agonists in the treatment of endotoxin-induced ALL. Although all of the in vivo evidence was established in murine models, it will be an important challenge to translate these findings into a clinical setting. In addition, it will be critical to determine convenient pharmacological approaches to use A2BAR agonists and study potential side effects of these compounds, for example, with regard to blood pressure, heart rate (52), or platelet function (53).

Disclosures
The authors have no financial conflicts of interest.

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