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

Ulrich Schingnitz, Katherine Hartmann, Christopher F. MacManus, Tobias Eckle, Stephanie Zug, Sean P. Colgan, and Holger K. Eltzschig

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: 5271–5279.

Sepsis is a serious medical condition characterized by a whole-body inflammatory state (called systemic inflammatory response syndrome) and the presence of a known or suspected infection. The body develops this generalized inflammatory reaction as a response to microbes or microbial toxins (such as LPS) circulating in the blood. In the United States, sepsis is the leading cause of death in critically ill patients. As such, sepsis develops in 750,000 people annually, resulting in >210,000 mortalities (1). Mortality rates associated with severe sepsis and septic shock are 25–30% and 40–70%, respectively (2). One of the main problems for patients with sepsis is the development of respiratory failure. Indeed, up to 40% of patients with sepsis go on to develop respiratory failure in the form of acute lung injury (ALI) or its more severe form acute respiratory distress syndrome (3). ALI is characterized by bilateral pulmonary edema, and severe hypoxia and is considered the leading cause of death in patients suffering from sepsis (4). At present, therapeutic approaches for patients suffering from septic lung injury are mainly symptomatic (1, 3). Therefore, the search for novel and specific therapies to prevent or treat sepsis-associated respiratory failure and endotoxin-induced ALI is an area of intense investigation.

Previous studies indicated that the generation of the extracellular signaling molecule adenosine plays a role in inflammatory cell trafficking and lung inflammation during endotoxin-induced lung injury (5). Adenosine is generated in the extracellular space via enzymatic phosphohydrolysis from its precursor molecules (6). This two-step reaction is under the enzymatic control of two enzymes located on the extracellular surface—the ecto-apyrase (conversion of ATP/ADP to AMP, CD39) (7) and the ecto-5'-nucleotidase (conversion of AMP to adenosine, CD73) (8). Studies in LPS-induced ALI and lung inflammation demonstrated that gene-targeted mice for CD39 or CD73 show a more severe degree of lung inflammation than their corresponding controls (5). These studies indicate a potential role for extracellular adenosine signaling in LPS-induced lung injury. Extracellular adenosine can signal through four different adenosine receptors (ARs), the A1AR, A2AR, A2BAR, and A3AR. Previous studies also showed that the dominant adenosine receptor in the lungs is the A2BAR (9) with its expression predominantly on pulmonary epithelia (10), vascular endothelia (11–14), or inflammatory cells (11, 14). Functional studies implicated the A2BAR in hypoxia-driven pulmonary edema (11) or lung inflammation during mechanical ventilation (15). Moreover, a recent study demonstrated a key role for A2BAR-dependent attenuation of hypoxia-driven inflammation of mucosal organs, including the lungs (16). On the basis of these studies, we hypothesize that the A2BAR could represent a potential therapeutic target for endotoxin-induced ALI. Therefore, we used a combination of pharmacological and genetic approaches to determine the role of the A2BAR in lung inflammation in LPS-induced lung injury.
Materials 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 cultured as described previously (17–20). Primary pulmonary endothelial cells (HMVEC-L; Lonza Walkersville, Walkersville, MD) were cultured under the supplier’s instructions. Cells were grown to full confluency and stimulated with 10 ng/ml LPS from Sigma-Aldrich (Taufkirchen, Germany), 10 ng/ml IL-1β (PeproTech, Heidelberg, Germany), 70 ng/ml IL-4 (PeproTech), and 20 ng/ml IL-6 (PeproTech) 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 according to the manufacturer’s instructions. Cells were then treated with either 0.5 μM PGE2 or vehicle (0.015% ethanol) for 4 h at 37°C. Subsequently, cells were then 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 in 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-anti-IgG (Santa Cruz Biotechnology) and donkey anti-goat IgG (Santa Cruz Biotechnology). Labeled bands from washed blots were developed with a 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. Samples were incubated with 5,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) to perform nuclear counterstaining.

**Pulmonary quantification of neutrophils and pulmonary edema**

Pulmonary infiltration by polymorphonuclear neutrophils (PMNs) was quantified by enzymatic assay for the azurophil 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−/− 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→WT and A2BAR−/−→A2BAR−/− 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 × 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 137Cs source. Immediately after irradiation, 1 × 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 monocyteic cells and ∼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 ± 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

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]-1α) (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-1β, IL-4, and IL-6 over a time-course of up to

![FIGURE 1. A2BAR expression following inflammatory stimulation in vitro. A–D, Pulmonary epithelial cells (A549) or vascular endothelia (HMEC-1) were exposed to the indicated concentrations of inflammatory mediators, and A2BAR transcript levels were determined by real-time RT-PCR following 0–24 h of exposure time. Data were calculated relative to the internal housekeeping gene (β-actin) and are expressed as mean fold change compared with control (0 h of exposure) ± SD at each indicated time (n = 4). E, Degradation curve of A2BAR mRNA in HMEC-1 cells treated with PGE2 and challenged with DRB (n = 3; *p < 0.005 by ANOVA). F, A2BAR expression in primary pulmonary endothelial cells treated with IL-6 (HMVEC-L) (n = 4). G, Western blot analysis of A2BAR protein following 24 h of IL-6 stimulation at indicated concentrations. The same blot was stripped and reprobed for murine β-actin to control for loading conditions. One representative of three Western blots is displayed.](http://www.jimmunol.org/)

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24 h and assessed regulation of A2ABR expression by real-time RT-PCR (Fig. 1A–D). In fact, these studies revealed dramatic increases in A2BAR 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. 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 x400). One representative image from three pulmonic sections is displayed.
Pharmaceutical 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 pharmaceutical 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. Lungs were excised after 4 h, and inflammatory markers were determined. LPS treatment was associated with increases in pulmonary transcript levels of IL-1β, IL-6, 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 pharmaceutical inhibitor of the A2BAR synergistically enhances endotoxin-induced increases in lung inflammation during ALI.

Gene-targeted mice for the A2BAR develop a more severe phenotype in endotoxin-induced ALI compared with that of control animals

After having shown that pharmaceutical inhibition of the A2BAR is associated with increased lung inflammation following LPS inhalation, we next pursued studies in previously characterized mice gene-targeted for the A2BAR (11, 16, 21, 24). For this purpose, WT or A2BAR<sup>−/−</sup> mice matched in age, gender, and weight were exposed to aerosolized LPS for 30 min. Consistent with our studies of pharmaceutical A2BAR inhibition (Fig. 3), we found significantly augmented elevations of pulmonary transcript levels for IL-1β (Fig. 4A), IL-6 (Fig. 4B), or TNF-α (Fig. 4C) in A2BAR<sup>−/−</sup> mice following LPS exposure when compared with those of WT treated animals. Moreover, elevations of IL-6 protein levels following LPS treatment in WT mice were dramatically enhanced in A2BAR<sup>−/−</sup> mice (Fig. 4D). Similarly, LPS treatment-induced increases in lung water in WT mice were far more pronounced in A2BAR<sup>−/−</sup> mice (Fig. 4E). Furthermore, LPS-induced increases in MPO activity were enhanced in A2BAR<sup>−/−</sup> mice (Fig. 4F). To confirm these findings on a structural level, we histologically examined lungs of WT or A2BAR<sup>−/−</sup> mice following LPS exposure. Histological scoring revealed that A2BAR<sup>−/−</sup> mice exhibited a more severe pulmonary phenotype following LPS exposure than that of WT animals (Fig. 5A, 5B). Taken together, these studies indicate a more severe phenotype of LPS-driven lung injury following genetic deletion of the A2BAR and provide indirect evidence for a protective role of A2BAR signaling during acute lung inflammation.

LPS-induced lung injury in A2BAR bone marrow chimeric mice

After having demonstrated detrimental effects of genetic deletion or pharmaceutical inhibition of A2BAR signaling during endotoxin-induced ALI, we were interested to define the contributions...
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 endotoxin-induced ALI. As expected, A2BAR+/+ → A2BAR−/− chimeric mice showed a similar degree of LPS-induced increase in lung inflammation as that in WT mice, whereas A2BAR−/− → A2BAR+/+ mice showed a phenotype similar to that in A2BAR−/− mice (Fig. 6A). However, bone marrow chimera expressing the A2BAR exclusively on the pulmonary tissues (A2BAR−/− → A2BAR+/+ chimeric mice) had a significantly lower degree of LPS-induced IL-6 elevation or pulmonary edema than bone marrow chimera with exclusive A2BAR expression on the myeloid lineages (A2BAR+/+ → A2BAR−/−) (Fig. 6A, 6B). Taken together, these studies suggest an important contribution of pulmonary A2BAR signaling in endotoxin-induced ALI and pulmonary inflammation.

### 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 expected, treatment with BAY 60-6583 was associated with attenuated pulmonary myeloperoxidase elevations following LPS treatment (Fig. 7A) indicating diminished LPS-elicted inflammatory cell accumulation. Similarly, LPS-elicted 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.
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 less 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 weather 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 ALI. 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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