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A2B adenosine receptor signaling attenuates acute lung injury by enhancing alveolar fluid clearance in mice

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Although acute lung injury contributes significantly to critical illness, resolution often occurs spontaneously via activation of incompletely understood pathways. We recently found that mechanical ventilation of mice increases the level of pulmonary adenosine, and that mice deficient for extracellular adenosine generation show increased pulmonary edema and inflammation after ventilator-induced lung injury (VILI). Here, we profiled the response to VILI in mice with genetic deletions of each of the 4 adenosine receptors (ARs) and found that deletion of the A2BAR gene was specifically associated with reduced survival time and increased pulmonary albumin leakage after injury. In WT mice, treatment with an A2BAR-selective antagonist resulted in enhanced pulmonary inflammation, edema, and attenuated gas exchange, while an A2BAR agonist attenuated VILI. In bone marrow–chimeric A2BAR mice, although the pulmonary inflammatory response involved A2BAR signaling from bone marrow–derived cells, A2BARs located on the lung tissue attenuated VILI-induced albumin leakage and pulmonary edema. Furthermore, measurement of alveolar fluid clearance (AFC) demonstrated that A2BAR signaling enhanced amiloride-sensitive fluid transport and elevation of pulmonary cAMP levels following VILI, suggesting that A2BAR agonist treatment protects by drying out the lungs. Similar enhancement of pulmonary cAMP and AFC were also observed after β-adrenergic stimulation, a pathway known to promote AFC. Taken together, these studies reveal a role for A2BAR signaling in attenuating VILI and implicate this receptor as a potential therapeutic target during acute lung injury.

Introduction
Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are life-threatening disorders that can develop in the course of different clinical conditions such as pneumonia, acid aspiration, major trauma, or prolonged mechanical ventilation, and contribute significantly to critical illness (1). Recent epidemiological studies show that each year 75,000 patients in the United States alone die from ARDS (2). The pathogenesis of these diseases is characterized by influx of a protein-rich edema fluid into the interstitial and intraalveolar spaces as a consequence of increased permeability of the alveolar-capillary barrier (1) in conjunction with excessive invasion of inflammatory cells—particularly polymorphonuclear neutrophils (3–6). At present, only little is known about how to target the alveolar-capillary barrier function or leukocyte trafficking therapeutically during ALI. In fact, to our knowledge, no such strategies have been translated into clinical practice, and we are unaware of any specific therapy currently available beyond mechanical ventilation and other supportive measures (1).

Despite the large impact of ALI on morbidity and mortality in critically ill patients (1), many episodes are self-limiting and resolve spontaneously through unknown mechanisms. For example, patients undergoing major surgery requiring prolonged mechanical ventilation have an overall incidence of ALI between 0.2% and 5%, depending on the kind of surgery (7–9). In a recent study to identify endogenous pathways to attenuate ventilator-induced lung injury (VILI), we found that extracellular adenosine accumulates in the supernatant of pulmonary epithelia exposed to cyclic mechanical stretch in vitro (10). Similarly, pulmonary adenosine levels were elevated during mechanical ventilation in vivo (10). In fact, mice deficient in extracellular adenosine production showed dramatic increases in pulmonary edema and pulmonary inflammation when exposed to VILI (10). However, adenosine-dependent signaling pathways of lung protection during VILI remain unknown. As such, extracellular adenosine can signal through any of 4 G protein–coupled adenosine receptors (ARs), A1AR, A2AAR, A2BAR, and A3AR, which have all been implicated in tissue protection in different models of injury or inflammation (10–28). Therefore, we designed the present study to test the hypothesis that AR signaling plays an important role in VILI. For this purpose, we assessed VILI in genetic models for each individual AR. Because these studies pointed toward a pivotal role of A2BAR signaling, we confirmed these genetic studies using pharmacological approaches with specific A2BAR agonists and antagonists. Next, we created bone marrow chimeras to study A2BAR effects on hematopoietic versus non-hematopoietic cells. Finally, we pursued the contribution of A2BAR signaling to alveolar fluid clearance (AFC) during VILI. Taken together, these studies point toward a critical role of A2BAR signaling in attenuating VILI by dampening pulmonary inflammation, attenuation of pulmonary edema, and enhancement of amiloride-sensitive fluid transport mechanisms to dry out the lungs.

Nonstandard abbreviations used: AFC, alveolar fluid clearance; ALI, acute lung injury; AR, adenosine receptor; BAL, bronchoalveolar; ENaC, epithelial sodium channel; KC, keratinocyte-derived chemokine; MPO, myeloperoxidase; VILI, ventilator-induced lung injury.

Conflict of interest: The authors have declared that no conflict of interest exists.

Characterized mice that were gene-targeted for deficiency would significantly enhance pulmonary edema and lung protection during VILI (10), we hypothesized that AR attenuate survival during VILI. To this end, we exposed previously on previous studies showing extracellular adenosine generation in lung protection during VILI (10), we hypothesized that AR attenuate survival during VILI. To this end, we exposed previously characterized mice that were gene-targeted for A1AR (29), A2AAR (30), A2BAR (13), or A3AR (22) as well as corresponding age-, weight-, and gender-matched littermate controls to VILI. As shown in Figure 1, survival time during VILI or VILI-induced increases of albumin leakage into the bronchoalveolar (BAL) fluid were similar between A1AR−/−, A2AAR−/−, and A3AR−/− mice and their controls (Figure 1, A, B, and D). In contrast, survival time was significantly shortened in A2BAR−/− mice (Figure 1C; P < 0.001), while albumin leakage into the BAL fluid was dramatically increased (Figure 1C; P < 0.001). Taken together, these data indicate that specific genetic deletion of the A2BAR enhances murine VILI.

A2BAR−/− mice show increased pulmonary inflammation during VILI. To confirm our findings of enhanced VILI in A2BAR−/− mice, we next measured additional functional parameters. Here, we found that increases in lung water content (Figure 2A; P < 0.01) and attenuated gas exchange during VILI were enhanced in A2BAR−/− mice compared with controls (A2BAR−/−; Figure 2B; P < 0.01). Based on a recent study showing that A2BAR signaling attenuates pulmonary inflammation during acute hypoxia (31), we studied the influence of A2BAR signaling on pulmonary inflammation. As shown in Figure 2C, increases in pulmonary neutrophil numbers (as measured by myeloperoxidase [MPO]) with VILI were dramatically enhanced in A2BAR−/− mice (P < 0.001). Similarly, increases in proinflammatory cytokines (pulmonary TNF-α, IL-6, and keratinocyte-derived chemokine [KC, a mouse ortholog of human IL-8]) were enhanced (Figure 2, D–F; P < 0.001), while levels of the antiinflammatory cytokine IL-10 (Figure 2G; P < 0.001) were attenuated. Consistent with studies showing that pulmonary A2BAR signaling attenuates NF-κB activation via adenosine-mediated cullin-1 deneddylation (31), we pursued pulmonary NF-κB activation during VILI. Consistent with previous studies of mechanical stretch (32, 33), VILI was associated with a significant increase of pulmonary NF-κB activity (Figure 2H; P < 0.001). However, pulmonary NF-κB activity was increased at baseline (Figure 2H; P < 0.001), and VILI-induced increases of NF-κB activity were further enhanced in A2BAR−/− mice (Figure 2H; P < 0.001). While a number of mechanisms have been suggested to mediate the endogenous inactivation of NF-κB, each of these pathways converges on changes in the inducible degradation of IκBα (34). Thus, we determined whether A2BAR signaling influences IκB activity during VILI. In fact, VILI-associated decreases in pulmonary IκB activity were accentuated in A2BAR−/− mice (Figure 2I; P < 0.001), confirming a role of A2BAR signaling in attenuating IκB/NF-κB–dependent pulmonary inflammation during VILI. To confirm increased susceptibility of A2BAR−/− mice to lung injury on a histological level, we next examined lungs from A2BAR−/− and control mice after 180 minutes of ventilation at 45 mbar. As shown by VILI scores (Figure 2J) and in images from pulmonary sections (Figure 2K), histological signs of pulmonary injury associated with VILI were dramatically enhanced in A2BAR−/− mice. Taken together, these results reveal increased pulmonary inflammation and severity of VILI associated with genetic deletion of A2BAR.

Figure 1
VILI in mice gene-targeted for individual ARs. Previously characterized A1AR−/− (A; ref. 29), A2AAR−/− (B; ref. 30), A2BAR−/− (C), or A3AR−/− mice (D; ref. 22) or corresponding littermate controls were exposed to VILI, and survival times were determined during VILI. Mechanical ventilation was applied using pressure-controlled settings (inspiratory pressure of 35 mbar, inspired oxygen concentration 100%; respiratory rate and inspiratory/expiratory ratio were adjusted to maintain normal pH) until a cardiac standstill was observed in the surface electrocardiogram. Note the significantly attenuated survival of A2BAR−/− mice (C; P < 0.001, n = 8). Albumin concentration in the BAL fluid was determined by ELISA. For this purpose, the mice were mechanically ventilated using pressure-controlled ventilation with an inspired oxygen concentration of 100% for 180 minutes at 45 mbar. Note the significantly increased albumin concentration in the BAL fluid of A2BAR−/− mice (C; P < 0.001, n = 6).

Results
VILI is selectively enhanced in gene-targeted mice for the A2BAR. Based on previous studies showing extracellular adenosine generation in lung protection during VILI (10), we hypothesized that AR deficiency would significantly enhance pulmonary edema and attenuate survival during VILI. To this end, we exposed previously characterized mice that were gene-targeted for A1AR (29), A2AAR

3302
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Figure 2
VILI in mice gene-targeted for the A2BAR. (A–H) A2BAR−/− mice or littermate controls (A2BAR+/+) were mechanically ventilated using pressure-controlled ventilation with an inspired oxygen concentration of 100% over 180 minutes at 45 mbar. (A) Following ventilation, lungs were excised en bloc and weighed. Lungs were lyophilized for 48 hours, and lung water content (mg lung water/mg dry tissue) was determined. Results are presented as mean ± SD (n = 6). (B) To assess pulmonary gas exchange, blood gas analyses were performed by obtaining arterial blood via cardiac puncture. Analysis was performed immediately, and the ratio of the arterial partial pressure of oxygen (PaO₂) to the fraction of inspired oxygen (FiO₂) was determined. Results are presented as mean ± SD (n = 6). (C) Pulmonary neutrophil accumulation was quantified using a MPO assay. MPO activity was assessed using a spectrophotometric reaction with O-dianisidine hydrochloride. Absorbance at 450 nm was measured and reported as difference in OD (ΔOD) over 5 minutes. Results are presented as mean ± SD (n = 6). (D–I) TNF-α, IL-6, KC, IL-10, NF-κB, and IκBα levels were evaluated in lung tissue homogenates using a mouse ELISA. Results are presented as mean ± SD (n = 6). (J) For quantification of histological tissue damage by VILI following 180 min ventilation, VILI scores were assessed in A2BAR−/− or corresponding littermate control mice. Results are displayed as median (midline within boxes) and range (bars above and below boxes) (n = 4). (K) One of 4 representative photomicrographs (original magnification, ×200) stained with hematoxylin and eosin is displayed.
A2BAR is induced by mechanical ventilation. To further characterize A2BAR signaling in innate protection during VILI, we next pursued transcriptional consequences of mechanical ventilation on A2BAR expression patterns in vivo. For this purpose, we ventilated mice for 0 to 4 hours using pressure-controlled ventilation (100% inspired oxygen concentration, inspiratory pressure 35 mbar). After sacrificing the animals at the time points indicated in Figure 3, harvesting the lungs, and isolating RNA, we assessed transcript levels of ARs via real-time RT-PCR. Consistent with previous studies showing selective induction of the A2BAR with large-volume ventilation (35), these experiments revealed a prompt and selective induction of the A2BAR (Figure 3A; *P < 0.01*). In contrast, transcript levels of the A1AR or A3AR were repressed (*P < 0.05*; Figure 3A), while A2AAR transcript levels were unchanged with mechanical ventilation (Figure 3A). However, pharmacological studies of survival time or pulmonary albumin leakage with A1AR antagonist (DPCPX, 1 mg/kg i.p.) or A3AR antagonist (MRS1191, 1 mg/kg i.p.) revealed no functional changes in A1AR or A3AR inhibition (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI34203DS1). Consistent with previous studies (36), comparative analysis of pulmonary AR transcript levels at baseline showed that A2BAR had the highest expression levels (Figure 3B). This distribution was even further magnified following mechanical ventilation, resulting in 5.3-fold higher transcript levels of the A2BAR compared with the A2AAR (Figure 3B). Because a previous study had shown oxygen-dependent modulation of adenosine-signaling effects (37), we measured relative transcription levels of A2BAR following 4 hours of ventilation at different inspired oxygen concentrations (Supplemental Figure 2). However, we noted no transcriptional effects of the inspired oxygen concentration on A2BAR expression. Therefore, all further experiments were performed at 100% inspired oxygen concentration. Next we measured A2BAR protein by western blot analysis from whole lungs of ventilated mice and observed ventilation time-dependent increases in A2BAR protein (Figure 4B) with mechanical ventilation in WT and A2BAR–/– mice. IgG controls were used at identical concentrations and proteins were resolved by SDS-PAGE. Resultant western blots were probed with anti-A2BAR antibodies. To control for loading conditions, blots were stripped and reprobed for actin expression. One representative experiment of 3 is shown. (D) To examine the influence of mechanical ventilation on pulmonary A2BAR expression patterns, C57BL/6 mice were ventilated in a pressure-controlled setting over 0 h or 3 h (35 mbar inspiratory pressure, 100% inspired oxygen), and lungs were harvested at the indicated time points, shock frozen, and lysed, and proteins were resolved by SDS-PAGE. Resultant western blots were probed with anti-A2BAR antibodies. To control for loading conditions, blots were stripped and reprobed for actin expression. One representative experiment of 3 is shown. (D) To examine the influence of mechanical ventilation on pulmonary A2BAR expression patterns, C57BL/6 mice were ventilated in a pressure-controlled setting over 0 h or 3 h (35 mbar inspiratory pressure, 100% inspired oxygen concentration). Lungs were stained with antibodies for A2BAR. IgG controls were used at identical concentrations and staining conditions as the target primary antibodies (original magnification, ×400; *n = 4*).
pulmonary cAMP elevation during VILI. In fact, we achieved similar cAMP elevations by intratracheal application of a β2-adrenergic agonist (zinterol, 10⁻⁷ M, 300 μl intratracheally). This treatment was associated with elevated cAMP levels, attenuated albumin leakage, and improved survival (Figure 4, C–E). Taken together, these results suggest that the A2BAR is the predominant pulmonary AR during VILI. As such, A2BAR signaling effects result in elevated pulmonary cAMP levels and attenuation of ALI.

**Influence of A2BAR antagonist (PSB1115) and agonist (BAY 60-6583) treatment on VILI.** After having shown that the A2BAR⁻/⁻ mice developed profound pulmonary inflammation and tissue damage when exposed to VILI, we next sought to confirm these findings and other studies showing A2BAR on myeloid cells (15, 21), we generated A2BAR bone marrow–chimeric mice to study the contribution of pulmonary versus myeloid A2BARs to lung injury during VILI. As expected, A2BAR⁻/⁻→A2BAR⁻/⁻ chimeric mice showed a similar degree of VILI-induced increases in ALI as did wild type mice, while A2BAR⁻/⁻→A2BAR⁻/⁻ mice showed a similar phenotype as that of A2BAR⁻/⁻ mice (Figure 8, A–F). Interestingly, A2BAR⁻/⁻→A2BAR⁻/⁻ chimeric mice exhibited VILI-induced albumin leakage and increases in lung water, similar to A2BAR⁻/⁻ mice (Figure 8, A and B). In contrast, A2BAR⁻/⁻→A2BAR⁺/⁺ chimeric mice had attenuated VILI-induced albumin leakage and lung water, very similar to A2BAR⁺/⁺ mice (Figure 8A).
In contrast, inflammatory changes (increased MPO, TNF-α, IL-6, and KC) were intermediate in chimeric mice with the genetic deletion of either the myeloid A2BAR (A2BAR<sup>–/–</sup>→A2BAR<sup>+/+</sup>) or the pulmonary A2BAR (A2BAR<sup>++/–</sup>→A2BAR<sup>++/+</sup>), suggesting a combination of pulmonary and myeloid A2BAR signaling events in attenuating lung inflammation during VILI. Taken together, these studies suggest a dual role of the A2BAR during VILI in which pulmonary A2BARs mainly attenuate pulmonary edema.
and albumin leakage, while neutrophil accumulation and release of proinflammatory mediators are affected by both pulmonary and myeloid A2BARs.

Barrier protection of BAY 60-6583 requires pulmonary A2BARs, while antiinflammatory effects involve pulmonary and myeloid A2BARs. To further study the contribution of pulmonary and hematopoietic A2BARs to VILI, we next exposed A2BAR bone marrow–chimeric mice pretreated with BAY 60-6583 to VILI. Bone marrow–chimeric A2BAR mice expressing the A2BAR on tissues but not on their hematopoietic cells (A2BAR+/− → A2BAR+/+ mice) showed reduced BAL albumin leakage and lower wet/dry ratios when treated with BAY 60-6583 (2 mg/kg i.p.) compared with their vehicle-treated counterparts. In contrast, BAY 60-6583 treatment had no effect on albumin leakage or wet/dry ratios in A2BAR+/− → A2BAR−/− or A2BAR−/− → A2BAR−/− chimeric mice, suggesting that pulmonary A2BARs play a role in dampening pulmonary edema during VILI (Figure 9, A and B). In contrast, pulmonary inflammation (as assessed by pulmonary TNF-α, NF-κB, and IL-10 levels) were evaluated in lung tissue homogenates using murine ELISA (n = 6). (H) To assess pulmonary gas exchange, blood gas analyses were performed by obtaining arterial blood via cardiac puncture. The ratio of the arterial partial pressure of oxygen to the fraction of inspired oxygen was determined. Results are presented as mean ± SD (n = 6).

**Figure 6**
A2BAR agonist (BAY 60-6583) treatment. (A–C) A2BAR+/+, A2AAR−/−, and A2BAR−/− mice and their corresponding littermate controls were treated with 2 mg/kg BAY 60-6583 or vehicle 30 minutes prior to induction of anesthesia. Mechanical ventilation was begun, and mice were ventilated using pressure-controlled settings (inspiratory pressure of 45 mbar, 100% inspired oxygen concentration) until a cardiac standstill was observed in the surface electrocardiogram (P < 0.01, n = 8). In other studies, albumin concentrations in the BAL fluid were determined by ELISA after mechanical ventilation using pressure-controlled settings with an inspired oxygen concentration of 100% for 180 minutes at 45 mbar. (D) Pulmonary neutrophil sequestration was quantified using a MPO assay (n = 6). (E–G) TNF-α, NF-κB, and IL-10 levels were evaluated in lung tissue homogenates using murine ELISA (n = 6).
A2BAR signaling enhances amiloride-sensitive fluid transport during VILI. Based on previous studies showing a critical role of extracellular adenosine signaling in maintaining proper fluid levels in the lung alveoli (41–43) and our observation of increased AFC in gene-targeted mice for the CFTR (44) and with plasma epinephrine levels during VILI (Figure 11B). Similarly, increases in AFC with BAY 60-6583 treatment were not blocked by pretreatment with propanol, a nonselective β-adrenergic receptor antagonist (Figure 11C). However, as shown earlier, treatment with the β2-adrenergic agonist zinterol (10^{-7} M, 300 μl intratracheally) was associated with elevated cAMP levels, attenuated albumin leakage, and improved survival (Figure 4, C–E). Simultaneous treatment with zinterol and BAY 60-6583 were associated with increased elevations of cAMP and enhancement of AFC. In fact, ANOVA testing followed by Bonferroni correction revealed a statistically significant increase with the combination of zinterol and BAY 60-6583 (mice treated with zinterol alone had an AFC of 58.56% following high-pressure ventilation; mice treated with zinterol and BAY 60-6583 had an AFC of 64.3%; P < 0.01). While statistical analysis of these data determined that the groups were different, these differences were very small and may not be biologically significant (Figure 11D). Thus, segregation of the 2 pathways is complicated. The stimulation of A2BAR increases cAMP and PKA, as does β-agonist stimulation. While adenosine signaling resulted in Gs protein–coupled and cAMP-elevating A2BAR activation, β2-adrenergic agonist stimulation converged on a similar signaling cascade, thereby resulting in enhancement of AFC. Taken together, these studies provide evidence for a role of A2BAR in counterbalancing increases in pulmonary edema during VILI via activation of amiloride-sensitive fluid transport mechanisms.

Discussion

ALI contributes substantially to critical illness, as it occurs frequently (2) and carries a high mortality rate (1). Moreover, the only therapeutic interventions currently available are elimination of the causative agents and supportive therapy (1). Never-

Figure 7

A2BAR signaling during LPS-induced lung injury. (A and B) A2BAR−/− and A2BAR+/+ mice were exposed to LPS inhalation for 30 minutes. Twenty-four hours after LPS exposure, TNF-α in lung tissue homogenates and albumin concentration in the BAL fluid were determined using a murine ELISA (n = 6). (C and D) A2BAR−/− mice received 2 mg/kg BAY 60-6583 i.p. or were treated with vehicle 30 minutes prior to LPS inhalation. (C) TNF-α levels in lung tissues and (D) albumin concentrations in the BAL fluid (n = 6).
theless, in many instances, ALI resolves spontaneously through unknown mechanisms. Here, we used VILI as a model for ALI to study the role of extracellular adenosine signaling pathways in endogenous lung protection. Our results point toward a pivotal role of the A2BAR in attenuating VILI-induced increases in pulmonary edema and inflammation. Moreover, our in vivo studies of A2BAR bone marrow–chimeric mice suggest an important contribution in attenuating lung injury by pulmonary A2BARs by maintaining the capillary-alveolar barrier during VILI. In addition, studies of alveolar fluid transport suggest that A2BAR signaling attenuates pulmonary edema formation by enhancing amiloride-sensitive fluid transport mechanisms. These findings correlate with elevations of plasma epinephrine levels and elevations of cAMP, roughly equivalent to the effects of stimulation of A1AR-mediated pathways and activation of A2AAR-mediated activity, increasing sodium absorption from the alveolus. Chloride regulation of the activities of these 2 channels establishes the balance between fluid secretion and absorption from the alveolus — such as occurs during pulmonary edema or VILI — adenosine concentrations may decrease, resulting in loss of A1AR-mediated pathways and activation of A2AAR-mediated pathways (41, 42). A2AAR-mediated pathways increase ENaC expression and activity, increasing sodium absorption from the alveolus. Chloride follows sodium out of the cell to maintain electroneutrality, and, as a result of this sodium and chloride flux, fluid leaves the alveolus (i.e., AFC increases) (41, 42). It is important to point out that during large-volume ventilation, the A2BAR is transcriptionally induced (an almost 10-fold induction was observed in the present study, consistent with previous studies of nucleoside receptor knockouts) and becomes the predominant AR in the lungs. Such findings may explain our results for the role of A2BAR signaling in activation of AFC during VILI. While A2AAR and A2BAR signaling pathways both converge to increase pulmonary CAMP levels (43), a combined agonist of the A2AAR and A2BAR may represent the most effective pharmacological approach to utilizing adenosine pathways to dry out the lungs during pulmonary edema or VILI.
The observation of increased capillary-alveolar leakage with targeting of the A2BAR during VILI suggests a protective role of extracellular adenosine signaling for maintaining the pulmonary barrier function. This is consistent with previous studies showing barrier protection and antiinflammatory effects of extracellular adenosine signaling in models of acute injury (47), inflammation (21), and hypoxia (11, 48). Previous studies found different ARs responsible for tissue protection under such conditions (11–13, 16). For example, an elegant study in mice deficient in the A2AAR showed increased inflammation-associated tissue damage (18), providing evidence for A2AAR signaling as a mechanism for regulating acute inflammatory responses in vivo. In this study, a subthreshold dose of an inflammatory stimulus was associated with minimal tissue damage in control animals. A similar stimulus was sufficient to induce extensive tissue damage, more prolonged and higher levels of pro-inflammatory cytokines, and death in A2AAR+/– mice. Similar observations were made using other models of inflammation, liver damage, or bacterial endotoxin–induced septic shock, suggesting a role for A2AAR in the limitation and termination of tissue-specific and systemic inflammatory responses (18). Other studies of LPS-induced ALI confirmed a critical role for signaling through A2AArs in attenuating LPS-induced lung inflammation (4, 37). As such, another study revealed that hypoxia increased adenosine signaling, the effects of which are protective during LPS-induced lung injury (37). In fact, the administration of high oxygen concentration further aggravated lung injury in that study. The authors found that this was due to inhibition of hypoxia-elicited lung protective mechanisms (37). Along the same lines, a recent study has suggested a role for hematopoietic A2AAR signaling in attenuating LPS-induced lung injury (4). Consistent with the present work, in vitro studies have indicated a critical role of signaling through the A2BAR in the re-sealing of endothelia during transendothelial migration of neutrophils (49), particularly during conditions of limited oxygen availability (14, 15, 27, 50). Moreover, pharmacological inhibition or genetic deletion of the A2BAR during hypoxia exposure is associated with increased pulmonary edema and vascular leakage (11, 51). Interestingly, another study suggested protective roles of HIF activators in respiratory distress syndrome and bronchopulmonary dysplasia (52). Serendipitously, the pacemaker enzyme of extracellular adenosine generation, CD73 (53), and the A2BAR (54) are both selectively induced by HIF-1 and were both shown to be central to innate lung protection during VILI (10). The seemingly contradictory findings that the A2AAR appears to be critical to mediating LPS-induced lung injury (18, 37) but that the A2BAR plays a pivotal role in VILI might be explained by differences between LPS- and VILI-induced ALIs and in AR expression patterns during such conditions.

The present studies in bone marrow–chimeric mice suggest that mice genetically targeted for myeloid A2BARs exhibit no significant increase in albumin leak or lung water. On the other hand, the mice had a significant degree of pulmonary inflammation (Figure 8), as determined by increased levels of cytokines and MPO. These data indicate that increased levels of cytokines or neutrophils do not contribute to lung injury. This is consistent with previous studies showing that neutropenia does not prevent significant injury to the alveolar epithelium during ALI induced by hypoxia (55, 56). Studies of ambient hypoxia exposure (8% oxygen over 4 hours) of A2BAR bone marrow–chimeric mice revealed that hypoxia-associated vascular leakage was attenuated by vascular A2BAR signaling effects (11). In contrast, hypoxia-associated inflammation mainly involved myeloid A2BAR signaling (11).

In contrast to the present study of acute injury, studies investigating chronic pulmonary disease have identified a potential detrimental role of elevated adenosine levels (57–63). For example, levels of adenosine are chronically increased in the lungs of asthmatics (61) and correlate with the degree of inflammatory insult (62), suggesting a provocative role of adenosine in asthma or chronic obstructive pulmonary disease (63). In addition, adenosine deaminase–deficient (ADA-deficient) mice develop signs of chronic pulmonary injury in association with chronically elevated pulmonary adenosine levels. In fact, ADA-deficient...
mice die within weeks after birth from severe respiratory distress (64), and studies have suggested that attenuation of adenosine signaling may reverse the severe pulmonary phenotypes in ADA-deficient mice, suggesting that chronic adenosine elevation can affect signaling pathways that mediate aspects of chronic lung disease (59, 64). Nevertheless, the present studies revealed that A2BAR-dependent lung protection was not limited to VILI but also played a role in LPS-induced lung injury in which pulmonary inflammation was measured 24 hours following exposure to endotoxin.

In summary, the present study has combined pharmacological and genetic approaches to identify extracellular adenosine signaling through the A2BAR as part of innate protection from VILI. While inflammatory aspects of VILI are mediated through myeloid and pulmonary A2BAR signaling, VILI-induced defects in the alveolar-capillary barrier are attenuated by pulmonary A2BARs. In addition, A2BAR signaling contributes to enhancing alveolar fluid transport during VILI, suggesting that A2BAR agonist treatment is a “pulmonary diuretic” and protects the lungs during VILI by enhancing AFC.

**Methods**

*Murine mechanical ventilation.* All animal protocols were in accordance with the German guidelines for use of living animals and were approved by the Institutional Animal Care and Use Committees of the Tübingen University Hospital, the Regierungspäsidium Tübingen, or the University of Colorado Denver. Previously characterized mice gene targeted for the A1AR (kindly provided by Jurgen B. Schnerrmnan, NIH, Baltimore, Maryland, USA) (29), A2AAR (kindly provided by Catherine Ledent, Université Libre de Bruxelles, Brussels, Belgium) (30), or A3AR (kindly provided by Marlene Jacobson, Merck Research Laboratories, West Point, Pennsylvania, USA) (22) or corresponding littermate controls were matched according to sex, age, and weight. A2BAR-deficient mice were generated by Deltagen Inc. by replacing a 112-bp fragment (from base 156 to base 267) from the 1,076-bp protein–coding region of the A2BAR gene with a 6.9-kb IRES-lacZ reporter cassette. The targetted A2BAR gene mutation was generated by Deltagen Inc. by replacing a 112-bp fragment (from base 156 to base 267) from the 1,076-bp protein–coding region of the A2BAR gene with a 6.9-kb IRES-lacZ reporter and neomycin resistance cassette. The targeted A2BAR+/-/+A2BAR+/-; +/+ mice were treated with intratracheal BAY 60-6583 (1 μM) alone or in combination with amiloride (1 mM), and AFC was determined. *P < 0.01 compared with no amiloride. n = 8.* (E and F) Control mice were treated with intratracheal BAY 60-6583 (1 μM, 100 μl) and/or amiloride (1 mM) or vehicle following tracheotomy and initiation of mechanical ventilation. Mice were ventilated using pressure-controlled settings (inspiratory pressure of 45 mbar, 100% inspired oxygen concentration) until a cardiac standstill was observed in the surface electrocardiogram. Note that the longer survival time during VILI with A2BAR agonist treatment was abolished following amiloride treatment (n = 8).

**Figure 10**

Contribution of A2BAR signaling to AFC during VILI. (A) To determine whether A2BAR signaling affects pulmonary fluid transport, we measured AFC using a mechanically ventilated live mouse model. A2BAR-/- mice and littermate controls were mechanically ventilated in a pressure-controlled setting at 45 mbar for 0 to 180 minutes. AFC was measured by instilling 300 μl of iso-osmolar 0.9% NaCl solution with 5% BSA. Mechanical ventilation was continued for 30 minutes, and AFC was measured in the presence or absence of the ENaC inhibitor amiloride (1 mM). *P < 0.01 compared with no amiloride. n = 8.* (B) Influence of A2BAR antagonist PSB1115 on AFC during VILI. Following induction of VILI, control mice received PSB1115 (1 μM) alone or in combination with amiloride (1 mM), and AFC was determined. *P < 0.01 compared with no amiloride. n = 8.* (C) VILI was induced in A2BAR bone marrow–chimeric mice, and AFC was determined. *P < 0.01 compared with A2BAR+/-; A2BAR-/-, n = 8.* (D) Influence of A2BAR agonist BAY 60-6583 on AFC during VILI. Following induction of VILI, control mice received BAY 60-6583 (1 μM) alone or in combination with amiloride (1 mM), and AFC was determined. *P < 0.01 compared with no amiloride. n = 8.* (E and F) Control mice were treated with intratracheal BAY 60-6583 (1 μM, 100 μl) and/or amiloride (1 mM) or vehicle following tracheotomy and initiation of mechanical ventilation. Mice were ventilated using pressure-controlled settings (inspiratory pressure of 45 mbar, 100% inspired oxygen concentration) until a cardiac standstill was observed in the surface electrocardiogram. Note that the longer survival time during VILI with A2BAR agonist treatment was abolished following amiloride treatment (n = 8).
(13). ALI was induced with mechanical ventilation utilizing high inspira-
tory pressure levels (e.g., 45 mbar) during pressure-controlled ventilation. In
short, animals were anesthetized with pentobarbital (70 mg/kg i.p. for
induction; 20 mg/kg/h for maintenance) and placed on a temperature-
controlled heat table with a rectal thermometer probe attached to a
thermal feedback controller to maintain body temperature at 37°C. In
addition, all animals were monitored with an electrocardiogram (Hewlett
Packard). Fluid replacement was performed with normal saline, 0.05 ml/h
i.p. Tracheotomy and mechanical ventilation was performed as described
previously (10). In short, the tracheal tube was connected to a mechanical
ventilator (Siemens Servo 900C, with pediatric tubing). Mice were venti-
lated in a pressure-controlled setting at 45 mbar and the inspired oxygen
concentration on A2BAR transcription. In subsets of experiments, mice
were exposed to aerosolized LPS in a custom-built cylindrical chamber
connected to an air nebulizer (MicroAir; Omron Healthcare Inc.). The
outlet of the chamber was connected to a vacuum pump, and a constant
flow rate of 15 ml/min was ensured by a flow meter (Gilmont Instru-
ments). LPS from E. coli (Sigma-Aldrich) was dissolved in 0.9% saline
(500 μg/ml), and mice were allowed to inhale LPS for 30 min. Control
mice were exposed to saline aerosol.

Transcriptional analysis. To examine the influence of mechanical ventila-
tion on A2BAR transcript levels, C57BL/6j mice (Charles River Laborato-
ries) were ventilated in a pressure-controlled fashion using the settings
indicated in the figure legends. Mice were euthanized at the time points
indicated in the figure legends, and the remaining blood was removed from
the pulmonary circulation by injection of 1 ml of PBS into the right ven-
tricle. Lungs were excised and immediately frozen at –80°C until transcrip-
tional measurements during a ventilation period of 360 min revealed no
significant changes in heart rate or blood pressure (Supplemental Tables 1
and 2). In contrast, pulmonary gas exchange was significantly attenuated
(Supplemental Figure 4). These studies suggest that lung injury in this
model is predominantly confined to the lungs and death did not occur due
to significant hemodynamic effects.

LPS-induced lung injury. To study endotoxin-induced lung injury, mice
were exposed to aerosolized LPS in a custom-built cylindrical chamber
connected to an air nebulizer (MicroAir; Omron Healthcare Inc.). The
outlet of the chamber was connected to a vacuum pump, and a constant
flow rate of 15 ml/min was ensured by a flow meter (Gilmont Instru-
mments). LPS from E. coli (Sigma-Aldrich) was dissolved in 0.9% saline
(500 μg/ml), and mice were allowed to inhale LPS for 30 min. Control
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indicated in the figure legends, and the remaining blood was removed from
the pulmonary circulation by injection of 1 ml of PBS into the right ven-
tricle. Lungs were excised and immediately frozen at –80°C until transcrip-
tional profiling. For this purpose, total RNA was isolated using the total
RNA isolation NucleoSpin RNA II kit (Macherey & Nagel) as described
previously.p. Basal cAMP levels in lung tissue (500 μg/ml) were measured by instilling
iso-osmolar 0.9% NaCl solution with 5% BSA.

**Figure 11**
Influence of β2-adrenergic and/or A2BAR signaling on AFC during VILI. (A) Epinephrine plasma levels in A2BAR+/– and A2BAR−/− mice that were mechanically ventilated in a pressure-controlled setting at 45 mbar over 180 minutes. (B) Basal cAMP levels in lung tissue from A2BAR+/− mice that were treated with zinterol and/or
BAY 60-6583. (C and D) To determine β2-adrenergic and A2BAR signaling effects on pulmonary fluid transport, A2BAR+/– and A2BAR−/− mice were mechanically ventilated in a pressure-controlled setting at 45 mbar for 0 to 180 minutes. AFC was measured by instilling 300 μl of iso-osmolar 0.9% NaCl solution with 5% BSA. Mechanical ventilation was continued for 30 minutes,
and AFC was measured in the presence or absence of the nonselective β-adrenergic receptor antagonist propranolol (intratracheal instillation of 10−3 M propranolol combined with 3 mg/kg i.p. with or without BAY 60-6583 (10−3 M to the instilled fluid) or in the presence or absence of the β-adrenergic agonist zinterol (intratra-
cheal, 10−7 M). *P < 0.01 compared with no propranolol (C) or zinterol (D), by ANOVA with Bonferroni post-hoc test. n = 8. In subsets of experiments, either proprano-
lol or zinterol were added together with BAY 60-6583 10−3 M to the instilled fluid. §P < 0.01 compared with propranolol alone (C) or zinterol alone (D), by ANOVA
with Bonferroni post-hoc test. n = 8.
rabbit polyclonal antibody (epitope corresponding to amino acids 293–332 (Santa Cruz Biotechnology Inc.). The wash was repeated, and proteins determined as described previously (10, 13, 65).

Immediately after collection, BAL fluid was centrifuged at 1,000 g for 5 min at 4°C, and the supernatant was aliquoted for measurement of the albumin concentration. To obtain BAL fluid, the tracheal tube was disconnected from the mechanical ventilator and the lungs were lavaged 3 times with 0.5 ml of PBS. All removed fluid was centrifuged immediately, and the supernatant was stored at –80°C for later analysis.

To assess pulmonary gas exchange, blood gas analyses were performed in subsets of experiments by obtaining arterial blood via the left ventricle and blood was obtained via cardiac puncture. Analysis was performed immediately after collection with the I-STAT Analyzer (Abbott), and the arterial partial pressure of oxygen was measured, in addition to arterial partial carbon dioxide pressure and pH values.

Epinephrine ELISA from plasma. Blood was collected via puncture of the right ventricle. Plasma was stored at –80°C for later analysis after centrifugation (3,000 g, 5 min). Plasma epinephrine concentrations were measured using a competitive radioimmunoassay (Labor Diagnostika Nord) with a sensitivity of 11 pg/ml and intra- and interassay variabilities of 5% and 12%, respectively.

Measurement of pulmonary AMP. cAMP levels in lung homogenate were measured using a competitive immunoassay kit from R&D Systems.

Measurement of pulmonary PKA activity. PKA activity was determined using a PKA Kinase Activity Assay kit (Assay Designs).

Histopathological evaluation of ALI. Following ventilation at the settings indicated in the figure legends, the mice were euthanized and lungs were fixed by instillation of 10% formaldehyde solution via the tracheal cannula at a pressure of 20 mbar. Lungs were then embedded in paraffin and stained with hematoxylin and eosin. Two random tissue sections from 4 different lungs in each group were examined by a pathologist who was blinded to the genetic background and treatment of the mice. For each subject, a 5-point scale was applied: 0, minimal damage; 1 to >2, mild damage; 2 to >3, moderate damage; 3 to >4, severe damage; and 4+, maximal damage. Points were added together and are expressed as median ± range (n = 4).

Generation of A2BAR bone marrow–chimeric mice. To define the contribution of pulmonary or hematopoietic cell A2BAR to VILI, we generated bone marrow–chimeric mice in which bone marrow was ablated by radiation in WT mice (C57BL/6) followed by reconstitution with bone marrow derived from previously characterized mice gene-targeted for the A2BAR (13) and vice versa (A2BAR+/−A2BAR+/− and A2BAR−/−A2BAR−/−). Experiments with A2BAR+/−→A2BAR+/− and A2BAR−/−→A2BAR+/− mice served as con-
Briefly, to confirm efficiency of reconstitution, a mutated mouse strain, stem cells were incubated with R-phycoerythrin–conjugated anti-mouse Ly6G monoclonal antibody (neutrophils; BD Biosciences—Pharmingen), rat anti-mouse Ly6G monoclonal antibody (neutrophils; BD Biosciences—Pharmingen), rat anti-mouse Ly6G monoclonal antibody (neutrophils; BD Biosciences—Pharmingen), rat anti-mouse Ly6G monoclonal antibody (neutrophils; BD Biosciences—Pharmingen), rat anti-mouse B220 monoclonal antibody (B cells; BD Biosciences—Pharmingen), fluorescence isothiocyanate–conjugated rat anti-mouse CD8α monoclonal antibody (CD8α T lymphocytes; BD Biosciences—Pharmingen), or allophycocyanin-conjugated rat anti-mouse CD4 monoclonal antibody (CD4 T lymphocytes; BD Biosciences—Pharmingen) on ice for 30 min. CD11b+, CD8α+, and CD4+, B220+, and Ly6G+ cells were sorted by flow cytometry, and the percentage of cells expressing CD45.1 was determined in each population of cells (FACSscan, CellQuest; BD). Studies of pulmonary inflammation 8 wk following bone marrow transplantation showed no histological signs of inflammation or neutrophil accumulation (Supplemental Figure 7).

Measurement of AFC during VILI. AFC was assessed in vivo using a previously described technique adapted to measure AFC during VILI (41, 69). In short, mice were ventilated in a pressure-controlled ventilation mode at 45 mbar over 0 to 180 min using an inspired oxygen concentration of 100%. AFC was measured by instilling 300 μl of iso-osmolar (320 mOsm) 0.9% NaCl solution containing 5% acid-free BSA (Sigma-Aldrich) via the endotracheal catheter into the alveolar space over 10 s, followed by injection of 100 μl of air to achieve complete deposition of all fluid into the alveolar space. Mechanical ventilation was continued for 30 min, at which time the chest was opened to produce bilateral pneumothoraces to facilitate aspiration of the remaining alveolar fluid via the tracheal catheter. Protein concentration was measured using a modified Bradford assay (Bio-Rad). AFC was calculated using the following equation: AFC=1–(C0/C30), where C0 is the protein concentration of the instillate before instillation and C30 is the protein concentration of the sample obtained after 30 min of mechanical ventilation. Clearance is expressed as a percentage of total instilled volume (%/30 min).

Consistent with previous studies, basal mean AFC was 32% (41). This value was set to 1 (100%). AFC is shown as fold change compared with baseline AFC. In subsets of experiments, amiloride (10−3 M), PSB1115 (10−4 M), BAY 60-6583 (10−6 M), zintelor (10−3 M), the CFTR inhibitor (CFTRinh-172, 10−6 M), and propanolol (intratracheal instillation of 10−3 M propanolol combined with 3 mg/kg i.p.) were given together with the instillate. Amiloride sensitivity is reported as fold change of AFC compared with similarly treated mice not exposed to amiloride. In other studies, survival time following intratracheal application of BAY 60-6583 with or without amiloride was determined (see “Murine mechanical ventilation”).

Statistics. Lung injury score and survival data are given as median ± range. All other data are presented as mean ± SD from 4–6 animals per condition. We performed statistical analysis using the Student’s t test (2 sided, α < 0.05) or ANOVA to determine group differences. Lung injury score was analyzed with the Kruskal-Wallis rank test. Kaplan-Meier curves were compared using the Mantel-Haenszel log-rank test. P < 0.05 was considered statistically significant.

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