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Distinct Roles for the A_{2B} Adenosine Receptor in Acute and Chronic Stages of Bleomycin-Induced Lung Injury

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Abstract

Adenosine is an extracellular signaling molecule that is generated in response to cell injury where it orchestrates tissue protection and repair. Whereas adenosine is best known for promoting anti-inflammatory activities during acute injury responses, prolonged elevations can enhance destructive tissue remodeling processes associated with chronic disease states. The generation of adenosine and the subsequent activation of the adenosine 2B receptor ($A_{2B}R$) is an important processes in the regulation of both acute and chronic lung disease. The goal of this study was to examine the contribution of the $A_{2B}R$ in models of bleomycin-induced lung injury that exhibit varying degrees of acute and chronic injury. Intratracheal bleomycin exposure results in substantial acute lung injury followed by progressive fibrosis. In this model, genetic removal of the $A_{2B}R$ resulted in enhanced loss of barrier function and increased pulmonary inflammation, with few differences in indexes of pulmonary fibrosis. These results support an anti-inflammatory role for this receptor in this model of acute lung injury. In contrast, systemic exposure of mice to bleomycin resulted in modest acute lung injury together with progressive pulmonary fibrosis. In this model, the effects of $A_{2B}R$ removal on acute lung injury were negligible; however, there were substantial reductions in pulmonary fibrosis, supporting a profibrotic role for this receptor. $A_{2B}R$-dependent regulation of IL-6 production was identified as a potential mechanism involved in the diminished pulmonary fibrosis seen in $A_{2B}R$ knockout mice exposed to i.p. bleomycin. These studies highlight the distinct roles of $A_{2B}R$ signaling during acute and chronic stages of lung injury.

Acute lung injuries and chronic lung diseases are collectively the third leading cause of death in the United States (1). Treatment options for these pulmonary disorders are limited due in part to an incomplete understanding of the cellular and molecular pathways involved in the lung's ability to respond to various forms of injury. Adenosine is an extracellular signaling molecule that is generated in response to cell injury where it orchestrates tissue protection and repair (2). Whereas adenosine is best known for promoting anti-inflammatory activities during acute injury responses (3, 4), prolonged elevations in adenosine can enhance destructive tissue remodeling processes associated with chronic disease states (5, 6). Consistent with this paradigm, adenosine serves as an anti-inflammatory molecule in models of acute lung injury (7-10), whereas elevations in adenosine contribute to disease

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progression in models of chronic lung disease (11, 12). Thus, adenosine signaling may play an important role in regulating how the lung responds to injury, and targeting this pathway may provide avenues for enhancing the resolution of acute lung injury or halting the progression of chronic lung disease.

Adenosine exerts its activities by engaging cell-surface adenosine receptors. There are four adenosine receptors (A_1R, $A_{2A}R$, $A_{2B}R$, A_3R) that are widely distributed on inflammatory and stromal cells (13, 14). Recent studies have identified the adenosine 2B receptor ($A_{2B}R$) as an important target in the regulation of both acute and chronic lung disease; however, with opposing activities in these disorders. Agonists for the $A_{2B}R$ are being considered for the treatment of acute lung injury based on findings demonstrating that activation of this receptor can enhance pulmonary barrier function and dampen inflammation in models of acute lung injury (7, 8). In contrast, $A_{2B}R$ antagonists are being considered for the treatment of chronic lung diseases based on results demonstrating that blockade of this receptor can diminish airspace destruction and fibrosis (11). These findings emphasize that understanding the contribution of the $A_{2B}R$ to the regulation of processes associated with acute and chronic lung injury will be essential for guiding the use of $A_{2B}R$ agonists and antagonists for the treatment of these disorders.

Recent studies from our laboratory suggest that $A_{2B}R$ signaling represents an important pathway for balancing the resolution of acute lung injury versus the progression to chronic disease (6). Mice deficient in the purine catabolic enzyme adenosine deaminase (ADA) develop features of chronic lung disease in association with prolonged adenosine elevations (15). The prominent aspects of pulmonary disease seen in this model include macrophage and neutrophil rich inflammation, alveolar airspace destruction, and fibrosis (15, 16). Genetic removal the $A_{2B}R$ from ADA-deficient mice resulted in enhanced acute lung injury characterized by the loss of vascular–alveolar barrier function and enhanced neutrophil accumulation (17). However, treatment of these mice with a selective $A_{2B}R$ antagonist during chronic stages of disease resulted in diminished pulmonary inflammation, airspace destruction, and fibrosis (11). These findings suggest that the $A_{2B}R$ plays a protective role during acute stages of lung injury, whereas detrimental effects of this signaling pathway are manifested at stages where tissue fibrosis is prominent.

These findings support the hypothesis that the $A_{2B}R$ is anti-inflammatory during acute lung injury and profibrotic during chronic disease stages. The goal of the current study was to examine this hypothesis further by exploring the consequences of genetic removal of this receptor in models of bleomycin-induced lung injury. Exposing mice to this toxicant by direct intratracheal (i.t.) administration or systemic exposure resulted in pulmonary fibrosis that was preceded by varying degrees of acute lung injury. Mice exposed to i.t. bleomycin developed substantial acute lung injury followed by fibrosis. In this model, genetic removal of the $A_{2B}R$ resulted in enhanced acute lung injury, with only modest decreases in fibrosis. In contrast, systemic exposure of mice to bleomycin resulted in modest acute lung injury followed by fibrosis, and genetic removal of the $A_{2B}R$ in this model resulted in substantial reductions in pulmonary fibrosis in association with diminished IL-6 production. These studies highlight the distinct roles of $A_{2B}R$ signaling during acute and chronic stages of lung injury.

Materials and Methods

Generation and genotyping of mouse lines

Wide-type C57BL/6 mice ($A_{2B}R^{+/+}$) were purchased from Harlan Laboratories (Indianapolis, IN). $A_{2B}R$-deficient mice ($A_{2B}R^{-/-}$) congenic on a C57BL/6 background were generated and genotyped as described (18). Animal care was in accordance with

National Institutes of Health guidelines and the Animal Care Committee at the University of Texas Health Science Center at Houston. All animals were housed under strict containment protocols and maintained in ventilated cages equipped with microisolator lids. Serologies on cage littermates were negative for 12 of the most commonly seen viruses in mice. No evidence of fungal, parasitic, or bacterial infection was found.

Intratracheal bleomycin treatment

Avertin (250 mg/kg, i.p.) was used to anesthetize 10-wk-old female C57BL/6 $A_{2B}R^{+/+}$ or $A_{2B}R^{-/-}$ mice, and saline alone or 2.5 U/kg bleomycin (Teva Parenteral Medicines, Irvine, CA) diluted in 50 μl sterile saline was instilled intratracheally (19). Endpoints were examined at 7 and 21 d after exposure.

Intraperitoneal bleomycin treatment

Six-week-old male $A_{2B}R^{+/+}$ or $A_{2B}R^{-/-}$ mice were treated intraperitoneally with 0.035 U/g bleomycin (Teva Parenteral Medicines) diluted in 150 μl sterile PBS or PBS alone (20). Mice were treated twice weekly for 4 wk and were sacrificed on day 10 and 33.

Bronchoalveolar lavage and histology

Avertin was used to anesthetize mice, and airways were lavaged three times with 0.4 ml PBS, and 1 ml bronchoalveolar lavage (BAL) fluid was recovered. Total cell counts were determined using a hemocytometer and cellular differentials determined by cytospinning BAL aliquots onto microscope slides and staining with Diff-Quick (Dade Behring, Deerfield, IL). After lavage, the lungs were inflated with 10% buffered formalin at 25 cm of pressure and fixed at 4°C overnight. Lungs were dehydrated in ethanol gradients and embedded in paraffin, and 5-μm tissue sections were collected on microscope slides and stained with H&E (Shandon-Lipshaw, Pittsburgh, PA) or Masson's trichrome (EM Science, Gibbstown, NJ) according to manufacturer's instructions.

Immunostaining

Rehydrated slides were quenched with hydrogen peroxide (3%), followed by Ag retrieval for 30 min at 95°C (Dako, Carpinteria, CA). Next, endogenous avidin and biotin blocking was performed with a Biotin Blocking System (Dako). For neutrophil staining, slides were incubated with rat anti-mouse neutrophil Ab (AbD SeroTec, Raleigh, NC; 1:500 dilution) overnight at 4°C. For α smooth muscle actin (α-sma) staining, slides were processed with mouse on mouse blocking reagents and incubated with an α-sma mAb (clone 1A4; Sigma-Aldrich, St. Louis, MO; 1:1000 dilution) at 37°C for 1 h. For IL-6 staining, slides were incubated with rabbit anti-mouse Ab (Abcam, Cambridge, MA; 1:100 dilution) overnight at 4°C. ABC streptavidin reagents and individual appropriate secondary Abs were used followed by development with 3,3′-diaminobenzidine (Sigma-Aldrich) and methyl green counterstaining for neutrophils and vector red alkaline phosphatase and Gill's hematoxylin for α-sma.

Measurement of vascular permeability

Pulmonary vascular permeability was quantified by i.p. administration of Evans blue dye (0.2 ml of 0.5% in PBS) (17). Four hours after dye administration, mice were perfused with PBS, and lungs were harvested and dye extracted in formamide overnight at 55°C. Dye concentrations were quantified by measuring absorbance at 610 nm with subtraction of reference absorbance at 450 nm. The content of Evans blue dye was determined by generating a standard curve from dye dilutions. Vascular permeability was also monitored by quantifying the levels of serum albumin in lavage fluid using ELISA (ICL, Newberg, OR).

Fibrosis assessment

The Sircol assay (Biocolor, Carrick, U.K.) was used to quantify soluble collagen levels in BAL fluid. Ashcroft scores were determined on H&E-stained lung sections (21). Twenty fields per section were used to determine the Ashcroft score, and four mice per group for controls and six mice per group for bleomycin-treated mice were examined. All analysis was conducted in a blinded manner.

Fibrotic mediator measurements

IL-6 and active TGF-β1 levels were quantified in 50 μl clarified BAL samples using an ELISA kit from BD Biosystems (San Diego, CA) following the manufacturer's instructions.

Statistics

Results were analyzed using the Mann–Whitney *U* test for comparison between any two groups and the non-parametric equivalents of ANOVA for multiple comparisons. A *p* value ≤ 0.05 was considered to be significant.

Results

Differences in anti-inflammatory effects of the $A_{2B}R$ in i.t. and i.p. models of bleomycin-induced lung injury

Intratracheal bleomycin exposure in mice results in acute lung injury followed by progressive pulmonary fibrosis (19). The $A_{2B}R$ serves anti-inflammatory roles in several models of acute lung injury (7-9), and we therefore hypothesized that the $A_{2B}R$ plays a similar anti-inflammatory role in the acute injury seen after i.t. bleomycin exposure. To address this, BAL cell counts and differentials were examined at an acute stage (day 7) after a single 2.5 U/kg i.t. bleomycin exposure (Fig. 1*A*). There were significantly more inflammatory cells recovered from the BAL fluid of $A_{2B}R^{-/-}$ mice compared with that of $A_{2B}R^{+/+}$ mice after i.t. bleomycin exposure. This included increases in macrophages, lymphocytes, and particularly neutrophils. These results suggest that the $A_{2B}R$ plays an anti-inflammatory role after i.t. bleomycin exposure.

Repeated i.p. injections of bleomycin also result in pulmonary fibrosis (20). To examine the contribution of the $A_{2B}R$ to acute inflammation in this model, BAL fluid cellularity was examined on day 10 (Fig. 1*B*). There were substantial increases in inflammatory cells, largely macrophages, in the BAL fluid of mice injected with bleomycin; however, there were no differences in the total numbers of inflammatory cells or the cell differentials in the BAL fluid of $A_{2B}R^{+/+}$ and $A_{2B}R^{-/-}$ mice injected with bleomycin. These findings suggest that the $A_{2B}R$ does not serve an antiinflammatory role in the i.p. model of bleomycin-induced lung fibrosis.

Previous studies suggest that the $A_{2B}R$ plays an important role in regulating neutrophil infiltration during acute lung injury (7, 8, 17). To examine more closely the differences in pulmonary neutrophils in these models of bleomycin-induced lung injury, lung sections from $A_{2B}R^{+/+}$ and $A_{2B}R^{-/-}$ mice were stained with an anti-neutrophil Ab to localize these cells (Fig. 2). Results demonstrated enhanced numbers of neutrophils in the alveolar airspaces and septae of $A_{2B}R^{-/-}$ mice exposed to i.t. bleomycin (compare Fig. 2*A* and 2*B*). Increased neutrophil staining was not seen in the lungs of $A_{2B}R^{-/-}$ mice exposed to i.p. bleomycin (compare Fig. 2*C* and 2*D*). These findings are consistent with a selective neutrophil increase in the lavage of $A_{2B}R^{-/-}$ mice exposed to i.t. bleomycin (Fig. 2*E*). These results suggest that the $A_{2B}R$ serves an anti-inflammatory role in acute inflammatory stages in the i.t. bleomycin model and that these responses are not seen in the i.p. model of pulmonary fibrosis.

Differences in the contribution of the $A_{2B}R$ to vascular permeability in i.t. and i.p. models of bleomycin-induced pulmonary injury

Acute lung injury is commonly associated with the loss of alveolar–vascular barrier function, which can contribute to enhanced neutrophil infiltration (8). Previous studies have shown that the $A_{2B}R$ plays a protective role in the maintenance of pulmonary barrier function in models of acute lung injury (7, 8, 17). We hypothesized that the $A_{2B}R$ plays a similar role in the acute injury seen after i.t. bleomycin exposure and that this effect would be attenuated in the i.p. model of bleomycin exposure. To address this, Evans blue dye extravasations were quantified in the lungs of $A_{2B}R^{+/+}$ and $A_{2B}R^{-/-}$ mice at acute stages after either i.t. or i.p. bleomycin exposure (Fig. 3). Results revealed that there was enhanced vascular leakage in $A_{2B}R^{-/-}$ mice compared with that in $A_{2B}R^{+/+}$ mice after i.t. bleomycin exposure (Fig. 3*A*, 3*B*). In contrast, there was only mild loss of vascular permeability in $A_{2B}R^{+/+}$ mice after i.p. bleomycin exposure, and this was not enhanced in $A_{2B}R^{-/-}$ mice (Fig. 3*A*, 3*B*). These findings suggest that the $A_{2B}R$ plays an important role in the maintenance of pulmonary barrier function in the i.t. bleomycin model. Furthermore, the loss of pulmonary barrier function and the protective role of the $A_{2B}R$ are less apparent in the i.p. model of bleomycin exposure.

Pulmonary fibrosis in $A_{2B}R^{-/-}$ mice after i.t. bleomycin exposure

To examine the contribution of the $A_{2B}R$ to fibrosis in the i.t. bleomycin model, $A_{2B}R^{-/-}$ mice were exposed to bleomycin intratracheally, and indicators of fibrosis were assessed 21 d later. Neither $A_{2B}R^{+/+}$ or $A_{2B}R^{-/-}$ mice exposed to saline showed histological signs of lung injury (Fig. 4*A*, 4*C*). However, both $A_{2B}R^{+/+}$ and $A_{2B}R^{-/-}$ mice exposed to bleomycin exhibited histopathological features of pulmonary fibrosis including congestion and remodeling of the alveolar airspaces (Fig. 4*B*, 4*D*). To assess fibrosis in these mice further, the presence of myofibroblasts was examined using α-sma immunohistochemistry (Fig. 5*A*, 5*B*), and collagen deposition was examined by staining with Masson's trichrome (Fig. 5*C*, 5*D*). Lungs from both $A_{2B}R^{+/+}$ and $A_{2B}R^{-/-}$ mice exhibited extensive α-sma and trichrome staining in the alveolar airspaces after the i.t. administration of bleomycin. To quantify the degree of fibrosis, soluble collagen levels were examined in BAL fluid at day 21 (Fig. 5*E*). Both $A_{2B}R^{+/+}$ and $A_{2B}R^{-/-}$ mice exhibited increased levels of collagen; however, there was not a significant difference in collagen accumulation between these two genotypes. Ashcroft scoring was conducted to assess the overall degree of fibrosis. Results demonstrated a slight reduction in overall fibrosis in $A_{2B}R^{-/-}$ mice (Fig. 5*F*). These results suggest that there is little to no difference in the degree of pulmonary fibrosis between $A_{2B}R^{+/+}$ and $A_{2B}R^{-/-}$ mice exposed to bleomycin by an i.t. route.

Development of pulmonary fibrosis in the i.t. bleomycin model is closely linked to inflammatory changes; however, we did not see an enhancement in pulmonary fibrosis in $A_{2B}R^{-/-}$ mice despite increased inflammation on day 7. To investigate this further, we examined inflammation, vascular permeability, fibrosis, and fibrotic mediator production at various time points in the progression of this model. As shown earlier, there was enhanced neutrophilia and vascular permeability in $A_{2B}R^{-/-}$ mice at day 7 after i.t. bleomycin exposure (Fig 6*A*, 6*B*); however, differences in inflammation and vascular leak between genotypes diminished at day 14 and 21. Collagen levels increased with time in the model; however, there were not significant differences between $A_{2B}R^{+/+}$ and $A_{2B}R^{-/-}$ mice (Fig. 6*C*). Investigation of the key profibrotic mediators IL-6 and TGF-β1 revealed significant elevations in $A_{2B}R^{-/-}$ mice during acute stages of the model; however, levels were diminished during chronic stages (Fig. 6*D*, 6*E*). These results suggest that IL-6 and TGF-β1 are differentially regulated during acute and chronic stages of i.t. bleomycin exposure, a feature that might account for the lack of increased fibrosis in the lungs of $A_{2B}R^{-/-}$ mice despite enhanced inflammation during acute stages.

Reductions in pulmonary fibrosis in $A_{2B}R^{-/-}$ mice after i.p. bleomycin exposure

To assess the role of the $A_{2B}R$ in fibrosis in the i.p. model of bleomycin injury, mice were injected intraperitoneally with bleomycin twice a week for 4 wk, and pulmonary fibrosis was assessed a week after the last bleomycin injection (day 33). Mice exposed to PBS were used as controls and exhibited normal pulmonary histopathology (Fig. 7*A*, 7*C*). $A_{2B}R^{+/+}$ mice treated intraperitoneally with bleomycin exhibited histopathological features of pulmonary fibrosis (Fig. 7*B*), and $A_{2B}R^{-/-}$ mice demonstrated diminished histopathological evidence of pulmonary fibrosis (Fig. 7*D*). Consistent with this, $A_{2B}R^{+/+}$ mice treated intraperitoneally with bleomycin exhibited pronounced α-sma (Fig. 8*A*) and Masson's trichrome (Fig. 8*C*) staining, and these metrics of pulmonary fibrosis were reduced in $A_{2B}R^{-/-}$ mice (Fig. 8*B*, 8*D*). Quantification of collagen levels (Fig. 8*E*) and Ashcroft scores (Fig. 8*F*) confirmed that there was significantly less pulmonary fibrosis in $A_{2B}R^{-/-}$ mice exposed to bleomycin by an i.p. route. These findings suggest that the $A_{2B}R$ plays a profibrotic role in the i.p. model of bleomycin-induced pulmonary fibrosis.

$A_{2B}R$-dependent expression of IL-6 after bleomycin exposure

IL-6 is a pluripotent cytokine that has profibrotic activities and is associated with pulmonary fibrosis in animal models and humans (22-24). In addition, we and others have shown that adenosine can promote the expression of IL-6 through the $A_{2B}R$ (11, 25, 26). IL-6 expression was monitored to determine if the regulation of this profibrotic mediator represents a mechanism by which $A_{2B}R$ engagement regulates fibrosis in the i.p. model. IL-6 levels were elevated more than 10-fold in the BAL fluid of $A_{2B}R^{+/+}$ mice exposed to i.p. bleomycin (Fig. 9*A*). In contrast, elevations in IL-6 were not detected in BAL fluid from the lungs of $A_{2B}R^{-/-}$ mice exposed to i.p. bleomycin (Fig. 9*A*). Immunolocalization experiments demonstrated that IL-6 production was abundant in macrophages in the airspaces of $A_{2B}R^{+/+}$ mice exposed to i.p. bleomycin (Fig. 9*B*). Furthermore, IL-6 expression in these cells was diminished in $A_{2B}R^{-/-}$ mice exposed to i.p. bleomycin (Fig. 9*B*). These findings suggest that the $A_{2B}R$ is essential for the production of IL-6 from alveolar macrophages in this model.

Discussion

In the current study, we demonstrate that the genetic removal of the $A_{2B}R$ leads to enhanced acute lung injury in a model of i.t. bleomycin exposure supporting an anti-inflammatory role for this receptor by promoting barrier function in the lung. In contrast, when bleomycin was administered via an i.p. route where acute lung injury was limited, removal of the $A_{2B}R$ resulted in diminished pulmonary fibrosis, suggesting a profibrotic role for this receptor in chronic stages of disease. These findings support the hypothesis that adenosine signaling through the $A_{2B}R$ plays distinct roles during acute and chronic stages of lung injury.

Generation of adenosine after cellular damage is an important component of the injury response where it plays active roles in processes that protect cells and tissues, limit inflammation, and promote wound healing (4-6). Engagement of all four adenosine receptors is involved in some capacity in these anti-inflammatory and tissue-protective activities. The $A_{2B}R$ has a relatively low affinity for adenosine (2) and thus may serve regulatory roles in pathological environments where adenosine is elevated (2, 12). This is supported by observations that the $A_{2B}R$ is markedly upregulated after various injurious stimuli including those seen during acute (7, 8) or chronic (27-29) lung injury. Mechanisms by which the $A_{2B}R$ is upregulated include transcriptional activation by Hif-1α (30) and regulation by micro RNAs (31). The $A_{2B}R$ serves anti-inflammatory and tissue protective roles in many acute injury models including cardioprotection by ischemic preconditioning (32) and protection from renal ischemic injury (33), sepsis (34), and acute lung injury (7-9).

Exposure of mice to hypoxia (7), ventilator-induced lung injury (8), or endotoxin (9) leads to acute lung injury in association with adenosine production and $A_{2B}R$ upregulation. The $A_{2B}R$ plays a protective role in these models by promoting vascular–alveolar barrier function and limiting inflammation, including neutrophilia. This was demonstrated by the enhancement of acute lung injury in mice lacking the $A_{2B}R$ and by the protection of mice from injury after treatment with an $A_{2B}R$ agonist. These findings suggest that $A_{2B}R$ agonist may be useful in the treatment of acute lung injuries.

Findings in the current study demonstrate for the first time, to our knowledge, that the $A_{2B}R$ serves an anti-inflammatory and tissue-protective role in the classical i.t. model of bleomycin-induced lung injury. This model is characterized by acute lung injury followed by progressive but resolvable pulmonary fibrosis (19, 35, 36). $A_{2B}R$ knockout mice given i.t. bleomycin demonstrated enhanced loss of pulmonary barrier function and excessive infiltration of neutrophils into the lung. The acute damage to the pulmonary epithelium after direct instillation of bleomycin to the airways likely contributes to the loss of barrier function in this model, and our findings suggest that activation of the $A_{2B}R$ plays important roles in promoting barrier function after this direct chemical injury. The exact mechanisms by which $A_{2B}R$ promotes barrier function in the lung is not clear; however, recent studies demonstrate that this receptor can be rapidly induced in airway epithelial cells and endothelial cells by various inflammatory mediators produced after acute lung injury (9), suggesting that upregulation of this pathway is important for enhanced barrier protection during acute inflammation. It is also possible that antiinflammatory effects may be due to the direct engagement of the $A_{2B}R$ on neutrophils (37); however, additional studies are needed to demonstrate functionality of this receptor on this cell type.

Notably, $A_{2B}R$ knockout mice go on to develop pulmonary fibrosis in the i.t. model to a degree that is similar to or slightly less than that seen in wild-type mice. These results suggest that the $A_{2B}R$ is not necessary for the development of pulmonary fibrosis in this model. Similar findings were seen in mice lacking the ecto enzyme CD73, which is responsible for generating adenosine after bleomycin-induced lung injury (38). CD73 mice exposed to i.t. bleomycin did not exhibit elevations in pulmonary adenosine levels and consequently developed enhanced acute lung injury, a finding that is likely mediated by the $A_{2B}R$ based on the current study. However, CD73 knockout mice went on to develop enhanced fibrosis in conjunction with enhanced inflammation, a common feature of the i.t. bleomycin model (23, 39). Notably, $A_{2B}R^{-/-}$ mice did not develop enhanced fibrosis despite enhanced inflammation suggesting this receptor plays an important role in regulating key mediators that amplify the fibrotic response. Indeed, time course evaluation of the production of IL-6 and TGF-β1 demonstrated that the levels of these fibrotic mediators are elevated during acute stages in the lungs of $A_{2B}R^{-/-}$ mice but are significantly reduced during chronic stages where fibrosis ensues. Thus, the lack of fibrotic enhancement in the presence of increased inflammation is likely due to reduced levels of these important profibrotic molecules. These findings are consistent with those demonstrating that antagonism of the $A_{2B}R$ can reduce pulmonary fibrosis in adenosine-dependent lung injury in conjunction with the regulation of IL-6 and TGF-β1 during chronic stages of fibrosis (11). These findings also suggest that the $A_{2B}R$ may impact processes that are more closely associated with fibrosis such as direct responses of fibroblasts (40), the recruitment of fibrocytes (41), or enhancement of remodeling responses such as epithelial to mesenchymal transition (42) or endothelial to mesenchymal transition (43).

These possibilities are validated further by our findings in the i.p. model of bleomycin-induced pulmonary fibrosis. We found that wild-type mice exposed to repeated i.p. injections of bleomycin did not develop acute lung injury to the degree seen in the i.t. bleomycin model. Consequently, an enhancement of the loss of barrier function and

increased pulmonary inflammation were not seen in $A_{2B}R$ knockout mice exposed to i.p. bleomycin. However, $A_{2B}R$ knockout mice exhibited significantly less pulmonary fibrosis than that of wild-type mice, including diminished quantities of myofibroblasts and collagen deposition in the airspaces and reduced levels of soluble collagen. This supports the conclusion above that the enhanced pulmonary inflammation in the i.t. model resulting from the lack of the $A_{2B}R$ leads to promotion of $A_{2B}R$-independent fibrotic stimuli. Furthermore, our finding suggests that the $A_{2B}R$ serves profibrotic effects in the absence of over-riding consequences of acute lung injury.

Further evidence to support this claim comes from a study demonstrating antifibrotic properties of an $A_{2B}R$ antagonist in the i.t. bleomycin model (11). Notably, in this study, the $A_{2B}R$ an-tagonist was administered during the fibrotic phase of the model and was associated with decreased pulmonary inflammation and decreased numbers of myofibroblasts and collagen content. These results suggest that the profibrotic effects of the $A_{2B}R$ may be directly associated with myofibroblast activity. In support of this, the $A_{2B}R$ has been shown to be the most abundant adenosine receptor expressed on human pulmonary fibroblasts (40). Furthermore, $A_{2B}R$ expression is increased in pulmonary fibroblasts after hypoxia and contributes to elevations in α-sma levels in vitro through the increased production of the profibrotic cytokine IL-6 (40). In addition to direct affects on fibroblasts and myofibroblasts, the $A_{2B}R$ may have indirect influences on fibrosis. Profibrotic mediators such as IL-6, TGF-β1, and osteopontin are elevated in the lungs of humans and animal models with pulmonary fibrosis (22, 44-47). Studies in ADA-deficient mice have demonstrated adenosine-dependent elevations in these profibrotic mediators in macrophages that accumulate in the airways during chronic stages of disease (11). Exposure of these mice to an $A_{2B}R$ antagonist during these stages resulted in decreased expression of these profibrotic mediators in association with decreases in pulmonary fibrosis. In support of these findings, we demonstrate in the current study that the $A_{2B}R$ is essential for the production of IL-6 from alveolar macrophages in the airspaces of mice exposed to i.p. bleomycin. We propose that the diminished levels of IL-6 production from macrophages in the airspaces may play a role in the diminished fibrosis seen in this model. Thus, $A_{2B}R$-mediated production of profibrotic molecules from macrophages may contribute to fibrotic progression in disease. The mechanisms by which $A_{2B}R$ activation promotes IL-6 production in macrophages is not fully understood but likely involves the upregulation of $A_{2B}R$ mRNA levels in activated reparative macrophages found during chronic stages of disease. Potential mechanisms under investigation include induction of the $A_{2B}R$ by hypoxia and regulation by micro RNAs.

Consistent with these findings in mice, recent studies have shown that the expression of the $A_{2B}R$ is increased in surgical biopsy specimens from the lungs of patients with a particularly severe form of interstitial lung disease known as idiopathic pulmonary fibrosis (IPF) (48, 49). Moreover, expression levels of the $A_{2B}R$ increase with the severity of fibrosis in IPF patients (48, 49). Immunolocalization studies place the $A_{2B}R$ on airway epithelial cells (48), fibroblasts, and alternatively activated macrophages in the lungs of IPF patients (49). Moreover, ex vivo analysis of alternatively activated macrophages from IPF patients demonstrates that activation of the $A_{2B}R$ directly promotes IL-6 production from these cells (49), further supporting the notion that $A_{2B}R$-dependent production of fibrotic mediators from macrophages may represent a mechanism of disease amplification in pulmonary disorders where fibrosis is present.

In conclusion, our studies highlight that the $A_{2B}R$ serves distinct functions during acute and chronic stages of lung disease. The exact mechanisms involved are not known; however, the $A_{2B}R$ is expressed on key cell types involved in the regulation of pulmonary barrier function [endothelial cells (27) and airway epithelial cells (9, 50)] and on cells that impact the

progression of fibrosis [macrophages (11) and myofibroblasts (40)]. Continued efforts to define the contribution of the $A_{2B}R$ to key cellular processes involved in lung disease will be critical for the advancement of $A_{2B}R$-specific therapies for acute or chronic lung diseases. In this regard, the preclinical data in animal models are striking regarding either the use of $A_{2B}R$ agonist in acute lung injury (36) or $A_{2B}R$ antagonist during chronic stages of disease (11). However, it will be critical to understand the expression and activity of the $A_{2B}R$ in patients with acute lung injury and chronic lung disease to ensure proper treatments (6).

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Abbreviations used in this article

$A_{2B}R$	adenosine 2B receptor
ADA	adenosine deaminase
BAL	bronchoalveolar lavage
IPF	idiopathic pulmonary fibrosis
i.t.	intratracheal
α-sma	α smooth muscle actin

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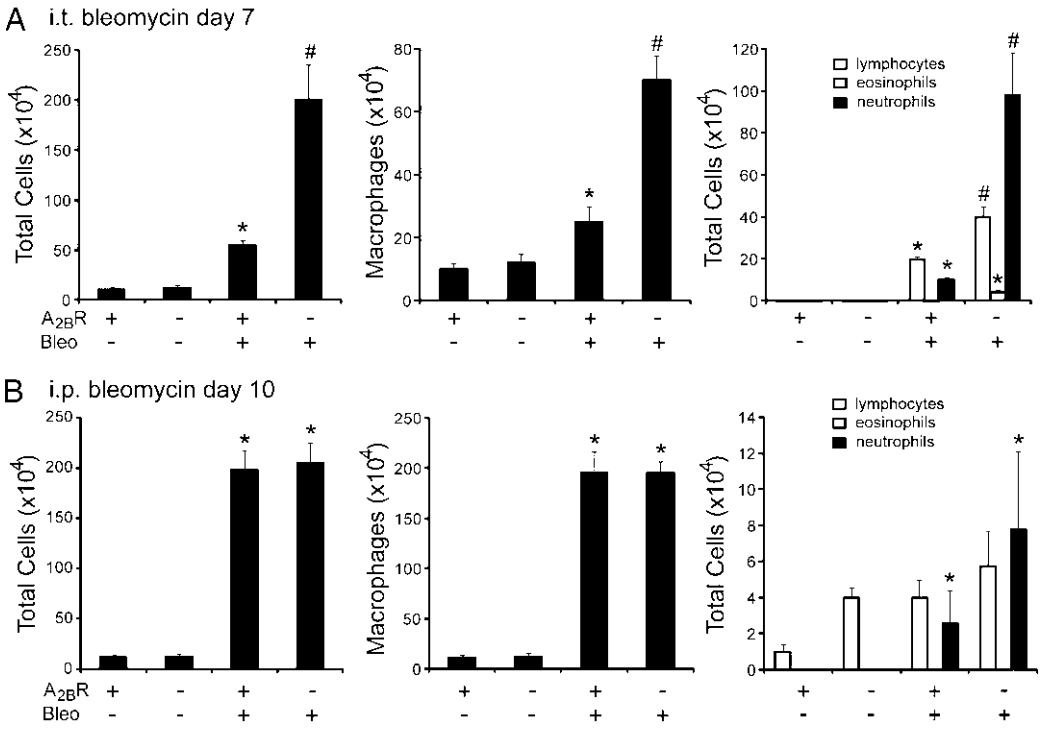

FIGURE 1.

Pulmonary inflammation after bleomycin exposure. BAL fluid was collected, and total cell numbers were determined. BAL cells were cytospun onto microscope slides and stained with Diff-Quick, allowing for determination of cellular differentials. *A*, BAL cell counts 7 d after i.t. bleomycin exposure. *B*, BAL cell counts 10 d after i.p. bleomycin exposure. Data are mean cell counts ± SEM. $*p \leq 0.05$ versus mice treated with saline or PBS; $^{\#}p \leq 0.05$ versus $A_{2B}R^{+/+}$ mice treated with bleomycin; $n = 8$.

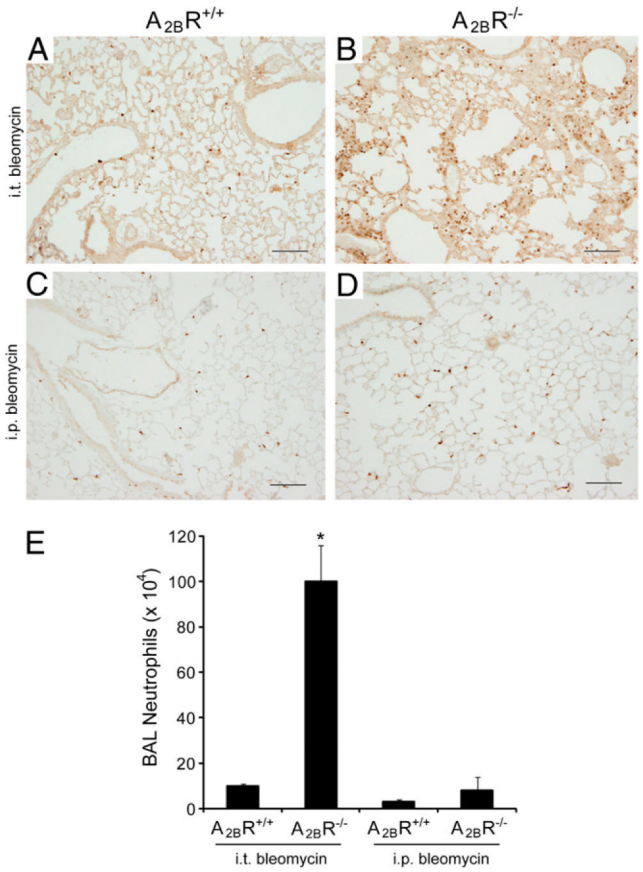

FIGURE 2.

Pulmonary neutrophils after bleomycin exposure. *A–D*, Lung sections from $A_{2B}R^{+/+}$ (*A*, *C*) and $A_{2B}R^{-/-}$ (*B*, *D*) mice collected 7 d after i.t. bleomycin exposure (*A*, *B*) or 10 d after the first i.p. exposure (*C*, *D*). Sections were reacted with an anti-neutrophil Ab to identify tissue neutrophils (brown signal). Sections are representative of six to eight different mice from each genotype. Scale bars, 200 μm. *E*, Neutrophil counts from BAL fluid collected 7 d after i.t. bleomycin and 10 d after i.p. bleomycin. Data are mean cell counts ± SEM. *$p \leq 0.05$ versus mice treated with saline or PBS.

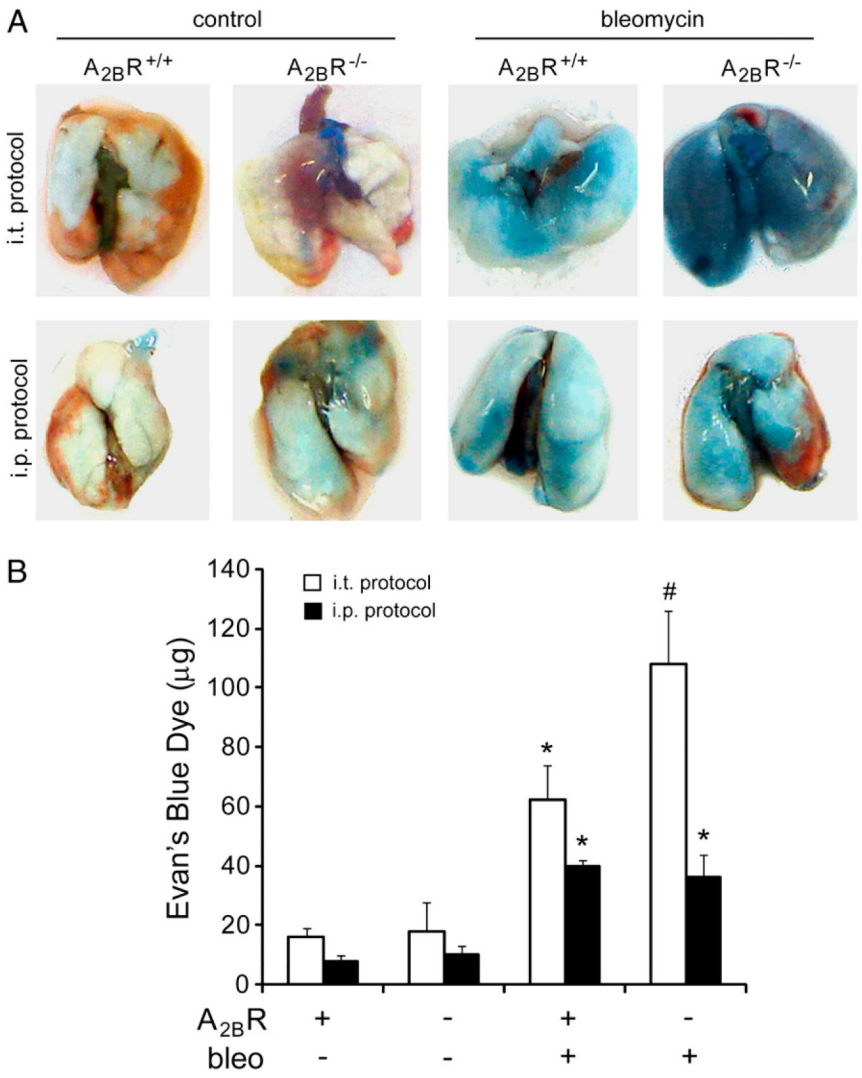

FIGURE 3.

Pulmonary vascular permeability after bleomycin exposure. Evans blue dye (0.2 ml of 0.5% in PBS) was delivered by i.p. injection to mice 7 d after i.t. bleomycin exposure or 10 d after the first i.p. bleomycin exposure. Four hours later, lungs were harvested and Evans blue dye concentrations examined. *A*, Representative images of lungs. *B*, Quantification of Evans blue dye levels in lungs. Data are mean dye amount ± SEM. $*p \leq 0.05$ versus mice treated with saline or PBS; $^{\#}p \leq 0.05$ versus $A_{2B}R^{+/+}$ mice treated with bleomycin; $n = 6$.

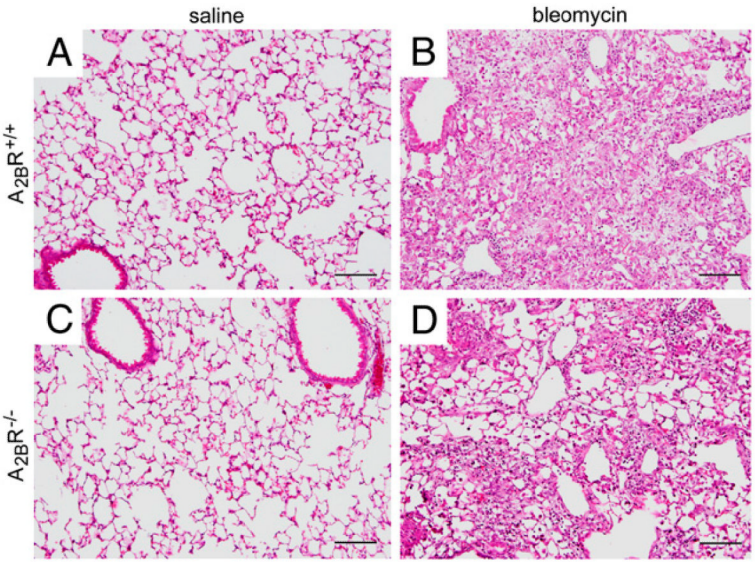

FIGURE 4.

Pulmonary histopathology after i.t. bleomycin exposure. Lungs were collected 21 d after i.t. bleomycin or saline exposure and prepared routinely for sectioning and H&E staining. *A*, Lung section from an $A_{2B}R^{+/+}$ mouse treated with saline. *B*, Lung section from an $A_{2B}R^{+/+}$ mouse treated with bleomycin. *C*, Lung section from an $A_{2B}R^{-/-}$ mouse treated with saline. *D*, Lung section from an $A_{2B}R^{-/-}$ mouse treated with bleomycin. Sections are representative of six to eight different mice from each genotype. Scale bars, 200 μm.

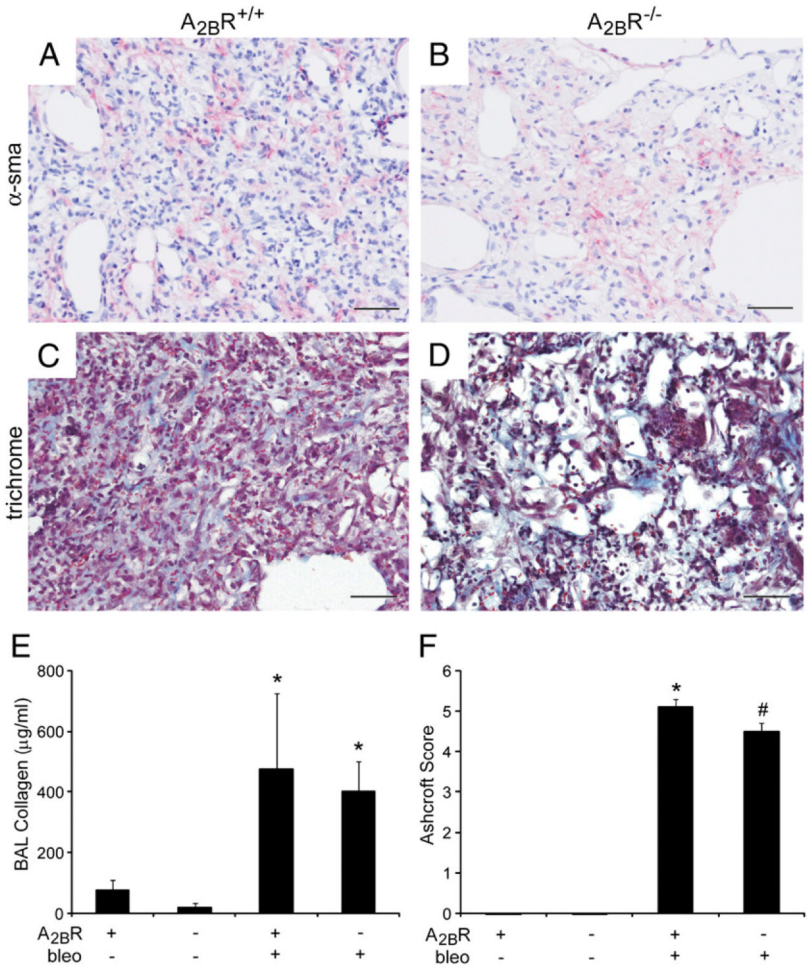

FIGURE 5.

Pulmonary fibrosis after i.t. bleomycin exposure. *A–D*, Lung sections from $A_{2B}R^{+/+}$ (*A*, *C*) and $A_{2B}R^{-/-}$ (*B*, *D*) mice collected 21 d after i.t. bleomycin exposure were subjected to α-sma immunostaining to identify myofibroblasts (pink signal in *A* and *B*) and stained with Masson's trichrome to identify collagen (blue signal in *C* and *D*). Sections are representative of six to eight different mice from each genotype. Scale bars, 100 μm. *E*, Soluble collagen protein levels in BAL fluid were quantified using the Sircol assay. Results are presented as mean collagen levels ± SEM. *F*, Ashcroft scoring was conducted to determine the extent of fibrosis. $^{*}p \leq 0.05$ versus ADA^{+} mice treated with saline; $^{\#}p \leq 0.05$ versus $A_{2B}R^{+/+}$ mice treated with bleomycin. $n = 6$ (saline treated), $n = 8$ (bleomycin treated).

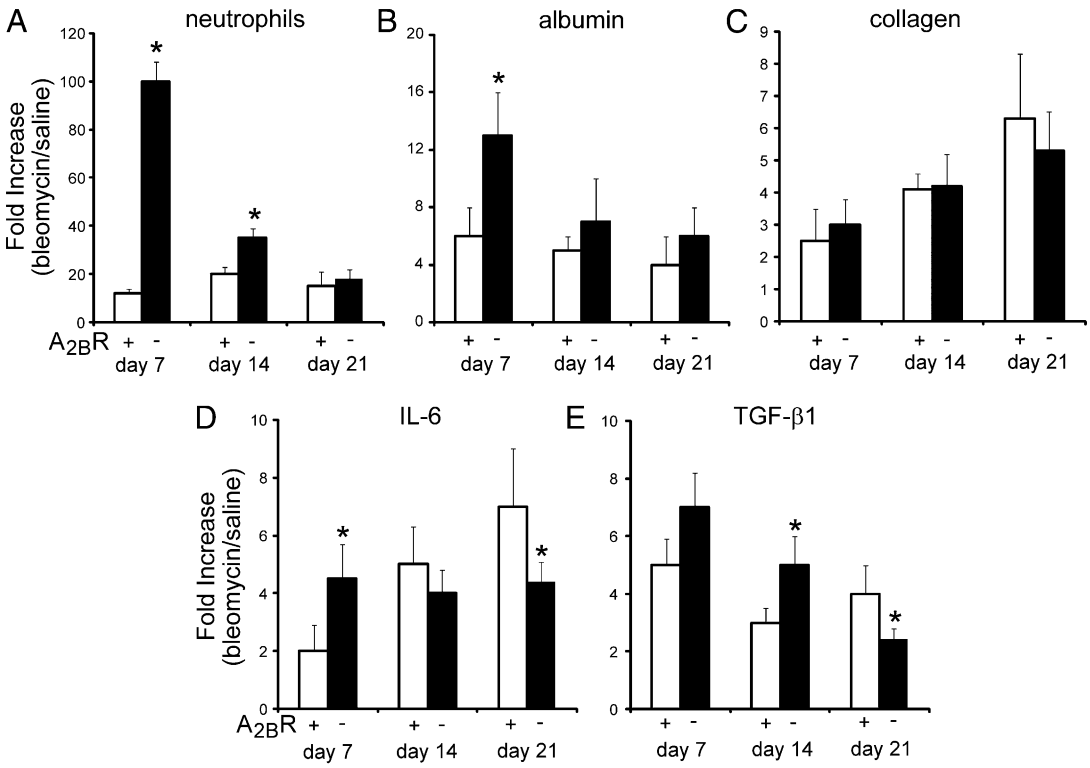

FIGURE 6.

Developmental analysis of pulmonary inflammation, permeability, and fibrosis after i.t. bleomycin exposure. BAL fluid was collected from $A_{2B}R^{+/+}$ (open bar) and $A_{2B}R^{-/-}$ (solid bar) mice at days 7, 14, and 21 after i.t. bleomycin exposure. *A*, Total BAL neutrophil counts. *B*, Vascular permeability as determined by levels of albumin in the BAL fluid. *C*, Soluble collagen levels determined by Sircol analysis. *D*, IL-6 protein levels in BAL fluid. *E*, Active TGF-β1 levels in BAL fluid. Values are presented as mean fold increases in bleomycin-exposed mice compared with saline-exposed mice ± SEM. $n = 4$ for each. $*p \leq 0.05$ comparing adjacent genotype.

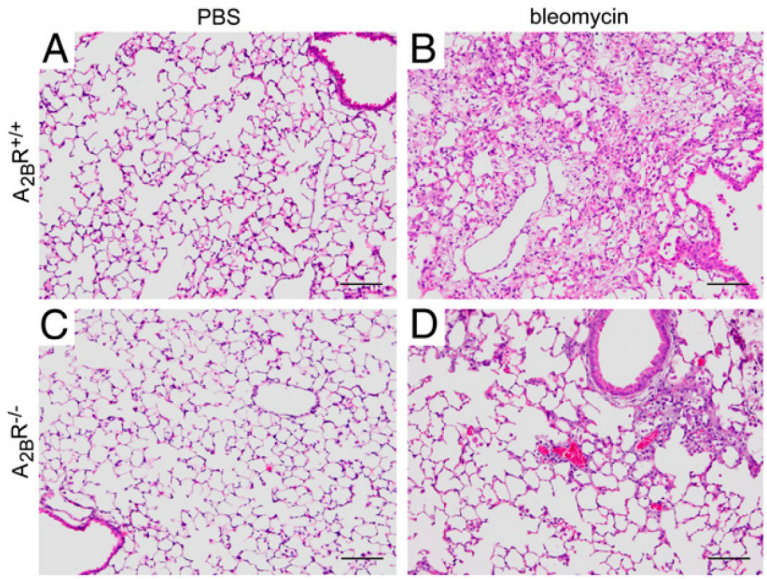

FIGURE 7.

Pulmonary histopathology after i.p. bleomycin exposure. Lungs were collected 33 d after the first bleomycin exposure and prepared routinely for sectioning and H&E staining. *A*, Lung section from an $A_{2B}R^{+/+}$ mouse treated with PBS. *B*, Lung section from an $A_{2B}R^{+/+}$ mouse treated with bleomycin. *C*, Lung section from an $A_{2B}R^{-/-}$ mouse treated with PBS. *D*, Lung section from an $A_{2B}R^{-/-}$ mouse treated with bleomycin. Sections are representative of six to eight different mice from each genotype. Scale bars, 200 μm.

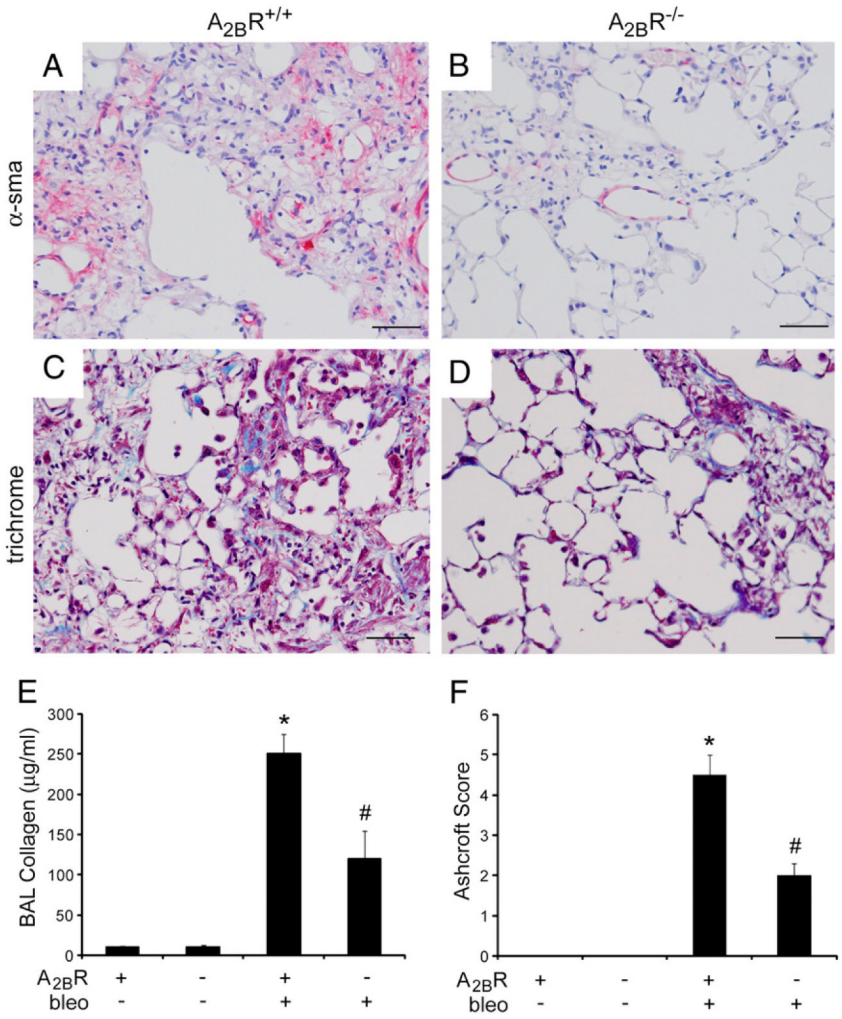

FIGURE 8.

Pulmonary fibrosis after i.p. bleomycin exposure. *A–D*, Lung sections from $A_{2B}R^{+/+}$ (*A*, *C*) and $A_{2B}R^{-/-}$ (*B*, *D*) mice collected 33 d after i.p. bleomycin exposure were subjected to α-sma immunostaining to identify myofibroblasts (pink signal in *A* and *B*) and stained with Masson's trichrome to identify collagen (blue signal in *C* and *D*). Sections are representative of six to eight different mice from each genotype. Scale bars, 100 μm. *E*, Soluble collagen protein levels in BAL fluid were quantified using the Sircol assay. Results are presented as mean collagen levels ± SEM. *F*, Ashcroft scoring was conducted to determine the extent of fibrosis. $*p \leq 0.05$ versus ADA^{+} mice treated with PBS; $^{\#}p \leq 0.05$ versus $A_{2B}R^{+/+}$ mice treated with bleomycin. $n = 6$ (saline treated), $n = 8$ (bleomycin treated).

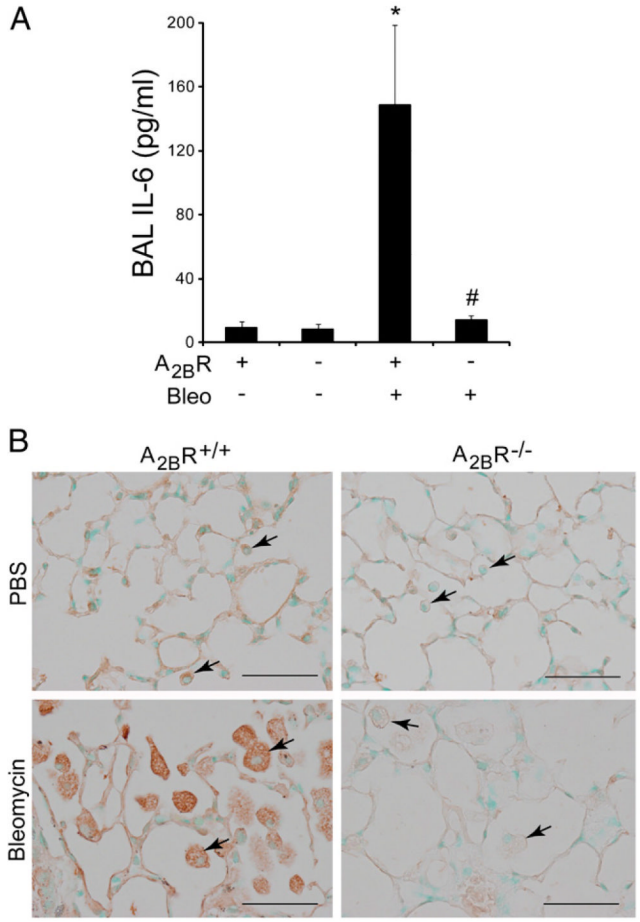

FIGURE 9.

IL-6 levels after i.p. bleomycin exposure. *A*, IL-6 levels were measured in BAL fluid collected from $A_{2B}R^{+/+}$ or $A_{2B}R^{-/-}$ mice on day 33 after i.p. PBS or bleomycin exposure using ELISA. Data are presented as mean pg/ml + SEM. $*p \leq 0.05$ versus ADA^{+} mice treated with PBS; $^{\#}p \leq 0.05$ versus $A_{2B}R^{+/+}$ mice treated with bleomycin. $n = 6$ (saline treated), $n = 8$ (bleomycin treated). *B*, Immunohistochemistry techniques were used to localize IL-6 expression in alveolar macrophages (arrows) in lung sections from $A_{2B}R^{+/+}$ or $A_{2B}R^{-/-}$ mice on day 33 after i.p. PBS or bleomycin exposure. Scale bars, 100 μm.