A₃ Adenosine Receptor Signaling Influences Pulmonary Inflammation and Fibrosis

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Adenosine is a signaling molecule produced during conditions that cause cellular stress or damage. This signaling pathway is implicated in the regulation of pulmonary disorders through the selective engagement of adenosine receptors. The goal of this study was to examine the involvement of the A₃ adenosine receptor (A₃R) in a bleomycin model of pulmonary inflammation and fibrosis. Results demonstrated that A₃R-deficient mice exhibit enhanced pulmonary inflammation that included an increase in eosinophils. Accordingly, there was a selective up-regulation of eosinophil-related chemokines and cytokines in the lungs of A₃R-deficient mice exposed to bleomycin. This increase in eosinophil numbers was accompanied by a decrease in the amount of extracellular eosinophil peroxidase activity in lavage fluid from A₃R-deficient mice exposed to bleomycin, an observation suggesting that the A₃R is necessary for eosinophil degranulation in this model. Despite an increase in inflammatory metrics associated with A₃R-deficient mice treated with bleomycin, there was little difference in the degree of pulmonary fibrosis. Examination of fibrotic mediators demonstrated enhanced transforming growth factor (TGF)- β 1 expression, but not a concomitant increase in TGF- β 1 activity. This was associated with the loss of expression of matrix metalloprotease 9, an activator of TGF- β 1, in alveolar macrophages and airway mast cells in the lungs of A₃R-deficient mice. Together, these results suggest that the A₃R serves antiinflammatory functions in the bleomycin model, and is also involved in regulating the production of mediators that can impact fibrosis.

Keywords: pulmonary fibrosis; adenosine receptors; inflammation; eosinophil; extracellular matrix

Pulmonary fibrosis is a component of many interstitial lung diseases (1). These disorders are characterized by varying degrees of inflammation, aberrant fibroblast proliferation, and extracellular matrix deposition that result in distortion of lung architecture and compromised pulmonary function (1–4). Fibrosis is also a major component of airway remodeling responses seen in asthma (5). Although the various signaling pathways that mediate pulmonary inflammation and fibrosis have not been completely elucidated, emerging evidence suggests that pulmonary fibrosis likely results from abnormal repair responses in the damaged lung, including deregulated inflammation, angiogenesis, and matrix metabolism (1, 6). In this regard, examining the regulation

CLINICAL RELEVANCE

Pulmonary fibrosis is associated with many interstitial lung diseases. The current study suggests that A₃ adenosine receptor signaling regulates key aspects of pulmonary inflammation and fibrosis.

of inflammation and the production of fibrotic mediators by inflammatory cells has become important for understanding the progression and chronicity of pulmonary diseases where fibrosis is a detrimental component.

Adenosine is a signaling molecule that is produced in excess at times of cellular stress and/or damage. Consistent with this, adenosine levels are elevated in the lungs of patients with asthma and patients with chronic obstructive pulmonary disease (COPD) (7, 8), as well as in the lungs of mouse models exhibiting inflammation and remodeling characteristic of lung diseases such as asthma, COPD, and idiopathic pulmonary fibrosis (9–11). Extracellular adenosine regulates numerous cellular responses by engaging specific G protein-coupled adenosine receptors on the surface of target cells (12). Among these responses is the activation of pathways that are important in the regulation of wound healing and fibrosis (13–16). Moreover, adenosine signaling pathways are associated with both pro- and antiinflammatory actions that are dictated by specific adenosine receptor subtypes and their downstream signaling components (17–19). Accordingly, adenosine signaling is emerging as a major pathway involved in regulating the balance between tissue repair and/or fibrosis.

The A₃ adenosine receptor (A₃R) has emerged as an important regulator of pulmonary inflammation and airway remodeling. Its levels are elevated in patients with chronic lung disease (20), and in numerous models of pulmonary disease (9, 21, 22). In addition, there is evidence that A₃R signaling can influence inflammatory cell functions relevant to pulmonary disease. For example, engagement of the A₃R on mouse mast cells promotes mediator release (23, 24), and engagement of the A₃R on eosinophils exhibits both pro- (25) and antiinflammatory (20, 21, 26) activities. Similarly, there is evidence that A₃R engagement can serve pro- and antiinflammatory roles on macrophages and neutrophils (27–29). Thus, there is evidence for both antiinflammatory and proinflammatory effects of A₃R signaling that may be relevant to lung disease.

The ability of adenosine to regulate both inflammation and fibrosis, and the pleiotropic effects of A₃R on immune regulation, led us to hypothesize that the A₃R is involved in regulating aspects of inflammation and fibrosis after lung injury. To test this hypothesis, we used a model of bleomycin-induced lung injury to examine the involvement of A₃R signaling in pulmonary inflammation and fibrosis. A₃R-deficient mice exhibited enhanced inflammation in response to bleomycin exposure as compared with wild-type mice, suggesting an anti-inflammatory role of the A₃R in this model. Surprisingly, despite the increased inflammation, there

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was little difference in the degree of fibrosis seen in the lungs of wild-type and A_3R -deficient mice exposed to bleomycin. Examination of fibrotic mediators revealed enhanced levels of transforming growth factor (TGF)- β 1 production; however, there was not a concomitant increase in TGF- β 1 activity. This was associated with diminished expression of matrix metalloproteinase (MMP)-9, an important activator of latent TGF- β 1, in alveolar macrophages and mast cells. Results also revealed for the first time that the A_3R is needed for eosinophil degranulation in this model, which may also contribute to the effects seen on fibrosis. Collectively, these studies indicate that the A_3R serves antiinflammatory functions in the bleomycin model. In addition, this receptor plays an important role in regulating the levels of fibrotic mediators that may help to influence the severity of ensuing fibrosis.

MATERIALS AND METHODS

Mice

A_3R -deficient ($A_3R^{-/-}$) mice congenic on a C57Blk6 background were obtained from Marlene A. Jacobson (Merck Research Labs, West Point, PA) (23), and wild-type C57Blk6 mice ($A_3R^{+/+}$) were obtained from Harlan (Indianapolis, IN). Animal care was in accordance with institutional and NIH guidelines, and all procedures were approved by the University of Texas Health Science Center at Houston Animal Welfare Committee. Mice were housed in ventilated cages equipped with micro-isolator lids and maintained under strict containment protocols. No evidence of bacterial, parasitic, or fungal infection was found, and serology on cage littermates was negative for 12 of the most common murine viruses.

Bleomycin Exposures

Eight- to 10-week old female $A_3R^{+/+}$ or $A_3R^{-/-}$ mice were anesthetized with avertin (250 mg/kg, intraperitoneally), and 3.5 U/kg bleomycin (Blenoxane; Bristol-Myers Squibb, Princeton, NJ) diluted in 50 μ l sterile saline or saline alone was instilled intratracheally. Endpoints were examined at 1, 3, 7, 14 and 21 days after challenge.

Histology and Immunostaining

Mice were anesthetized, and lungs lavaged three times with 0.4 ml PBS. Total cell counts were determined using a hemocytometer, and cellular differentials (300 cells/sample) were conducted on cells cytospun onto microscope slides and stained with Diff-Quick (Dade Behring, Newark, DE). Lungs were then infused with 10% buffered formalin at 25 cm of pressure and fixed overnight at 4°C. Fixed lungs were dehydrated and embedded in paraffin. Sections (5 μ m) were collected on microscope slides, rehydrated through graded ethanols to water then stained with hematoxylin and eosin (H&E; Shandon-Lipshaw, Pittsburgh, PA), Masson's trichrome or 0.1% toluidine blue.

For immunostaining, rehydrated slides were quenched with 3% hydrogen peroxide, antigen retrieval performed (Dako Corp. [Glostrup, Denmark] or Zymed pepsin [Zymed, San Francisco, CA]), and endogenous avidin and biotin blocked with a Biotin Blocking System (Dako Corp). Slides were incubated with primary antibody: α -smooth muscle actin (α -sma, 1:1,000 dilution [Sigma, St. Louis, MO] overnight at 4°C after processing sections with Mouse on Mouse Kit [Vector Laboratories, Burlingame, CA]), rabbit anti-mouse major basic protein 1 (mMBP-1, 1:1,000 dilution, 1 h at room temperature), or goat anti-mouse MMP-9 (1:50 dilution, 1 h at room temperature; R&D Systems, Minneapolis, MN). Sections were incubated with ABC Elite Streptavidin reagents and appropriate secondary antibodies, then developed with 3,3'-diaminobenzidine (Sigma-Aldrich) and counterstained with methyl green. Quantification of peribronchial eosinophils was assessed by analysis of 10 fields at $\times 20$ magnification. Eosinophils were counted using Image Pro Plus 4.0 image analysis software (Media Cybernetics, Silver Spring, MD). Immunofluorescence was conducted using goat anti-mouse MMP-9 (1:50 dilution, 1 h at room temperature; R&D Systems) followed by Alexa Fluor 488 (1:1,000, 1 h at room temperature; Invitrogen, Carlsbad, CA). Slides were coverslipped with Vectashield (Vector Laboratories) mounting medium containing DAPI to visualize nuclei.

Eosinophil Peroxidase Assay

Eosinophil peroxidase (EPO) activity present in 75 μ l of undiluted lavage fluid samples was detected after incubation in a 96-well plate

with equal volumes of substrate solution (50 mM Tris-HCl, pH 8, 0.1% Triton X-100, 8.8 mM H_2O_2 , 10 mM o-phenylenediamine [OPD], 6 mM KBr) at 37°C for 30 minutes. Duplicate samples also containing 10 mM of the peroxidase inhibitor resorcinol (1,3-Benzenediol; Sigma-Aldrich) were used as controls for the specificity of these reactions. The reaction was stopped with 50 μ l 4N H_2SO_4 , and OD values were read at 490 nm and EPO content given as OD/ml.

Analysis of mRNA

Mice were killed and the lungs were rapidly removed and frozen in liquid nitrogen. RNA was isolated from frozen lung tissue using TRIzol Reagent (Life Technologies, Inc., Gaithersburg, MD). RNA samples were then DNase-treated (DNaseI; Invitrogen) and subjected to quantitative RT-PCR. The primers, probes and procedures for the PCR reactions were as described previously (30, 31). Values were normalized to 18S ribosomal RNA and are presented as mean normalized transcript levels using the comparative C_t method ($2^{-\Delta\Delta C_t}$) (32).

MMP, Tissue Inhibitor of Metalloproteinase-1, and TGF- β 1 Assays

Gelatinolytic activity in bronchoalveolar lavage (BAL) fluid was assessed by zymography based on standard methods (33). Briefly, BAL fluid was subjected to 10% SDS-PAGE on gels containing 0.02% gelatin. After electrophoresis, SDS was removed by washes in 2.5% Triton X-100, before incubation at 37°C for 24 hours in developing buffer (50 mM Tris HCl, pH 8, containing 5 mM $CaCl_2$ and 0.02% NaN_3) with or without the MMP inhibitor 10 mM of 1'-10-phenanthroline (Sigma). Gels were fixed and stained with 50% methanol and 10% acetic acid that contained 0.3% wt/vol Coomassie blue R-250. MMP-9 and MMP-2 bands were identified by their molecular weight, and 10 μ l plasma from wild-type C57Bl/6 mice served as positive controls. Protein levels for MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1 were assessed in BAL fluid using Biotrak activity assay (MMP-9; GE Healthcare Life Sciences, Piscataway, NJ) and enzyme-linked immunosorbent assay (TIMP-1, R&D Systems). Active TGF- β 1 was measured using a mouse immunoassay kit (R&D Systems).

Fibrosis Assessment

The Sircol assay (Biocolor Ltd., Carrick, UK) was used to measure collagen in whole lung homogenates. Snap-frozen lungs were homogenized in 2 ml 0.5 M acetic acid containing pepsin (1:10 pepsin/tissue; Sigma-Aldrich) and then incubated overnight shaking at room temperature. Homogenates were spun at 10,000 rpm, and supernatant assayed for pepsin-soluble collagen. Ashcroft scores were determined on Masson's Trichrome-stained lung sections using a minor modification of the method outlined by Ashcroft and coworkers (34). Twenty fields per slide were examined using a two-person randomized blind study. At least six mice per group were examined.

Statistics

Values are expressed as means $\pm$ SEM. As appropriate, groups were compared by ANOVA, with follow-up comparisons between groups being conducted using Student's *t* test with a *P* value of < 0.05 denoting significant differences.

RESULTS

Pulmonary Inflammation in $A_3R^{-/-}$ Mice Treated with Bleomycin

$A_3R^{-/-}$ mice were exposed to bleomycin, and pulmonary inflammation was assessed 14 days later to examine the contribution of A_3R signaling to the pulmonary inflammation seen after bleomycin injury. There was no difference in appearance of the lungs of $A_3R^{+/+}$ and $A_3R^{-/-}$ mice treated with saline (Figures 1A and 1B), suggesting no obvious basal phenotype in the lungs of $A_3R^{-/-}$ mice. There was pronounced reorganization of the alveolar airways of $A_3R^{+/+}$ mice exposed to bleomycin (Figure 1C), with extensive fibrosis and inflammatory cell accumulation in the pulmonary interstitial spaces. $A_3R^{-/-}$ mice exposed to bleomycin also exhibited severe reorgani-

Figure 1. Pulmonary histopathology and inflammation. Mice were killed 14 days after saline or bleomycin exposure, and the lungs were lavaged and processed for hematoxylin and eosin staining. (A) Lung section from an A₃R^{+/+} mouse exposed to saline. (B) Lung section from an A₃R^{-/-} mouse exposed to saline. (C) Lung section from an A₃R^{+/+} mouse exposed to bleomycin. (D) Lung section from an A₃R^{-/-} mouse exposed to bleomycin. Photographs are representative of six to eight mice from each condition; scale bars = 100 μ m. (E) Total bronchoalveolar lavage (BAL) cell counts presented as mean cell counts $\times 10^4 \pm$ SEM. *Significant difference compared with saline treatment, #significant difference compared with bleomycin-treated A₃R^{+/+} mice ($P < 0.05$, $n = 6-8$).

zation of the alveolar spaces, with fibrosis and what appeared to be an enhancement of inflammatory cell foci (Figure 1D). These findings suggest that A₃R^{-/-} mice exhibit enhanced pulmonary inflammation after bleomycin exposure.

Mice were lavaged on Day 14 after exposure, and the number of inflammatory cells was quantified in BAL fluid to better characterize the pulmonary inflammation seen after bleomycin exposure. There was an 8-fold increase in the number of inflammatory cells recovered from the airways of A₃R^{+/+} mice exposed to bleomycin relative to that seen in A₃R^{+/+} mice exposed to saline (Figure 1E). Interestingly, the number of airway inflammatory cells was significantly higher in A₃R^{-/-} mice exposed to bleomycin. Analysis of cellular differentials demonstrated enhanced elevations in macrophages, neutrophils, and eosinophils in the airways of A₃R^{-/-} mice exposed to bleomycin (see Figure E1A in the online supplement). Examination of inflammatory mediators demonstrated enhanced expression of key cytokines and chemokines, including CCL2, IL-6, and CXCL1 (Table E1). These findings demonstrate that there is enhanced inflammation in the airways of A₃R^{-/-} mice 14 days after bleomycin exposure.

Eosinophil Trafficking and Activation in A₃R^{-/-} Mice Treated with Bleomycin

Mice were lavaged at various times after bleomycin exposure, and cellular differentials were determined to examine the ontogeny of

the pulmonary inflammation. There were no significant differences in the number of inflammatory cells recovered from the BAL of A₃R^{+/+} and A₃R^{-/-} on Day 1 after bleomycin exposure (Figures 2A and E1). There was a trend toward a developmental increase in neutrophils and macrophages in the airways of A₃R^{-/-} mice treated with bleomycin; however, only eosinophil numbers exhibited a significant increase during the later stages examined (Figure 2A). The enrichment of airway eosinophils in A₃R^{-/-} mice exposed to bleomycin prompted us to examine the levels of eosinophils within the lung parenchyma. Lung sections were stained with an antibody against mMBP-1 to identify tissue-infiltrating eosinophils (Figure 2B). More mMBP-1-positive cells were present within the interstitial spaces of lungs from bleomycin-treated A₃R^{-/-} mice, a feature that was verified by quantifying mMBP-1-positive cells in multiple tissue sections (Figure 2C).

To investigate the mechanisms leading to enhanced eosinophil recruitment to the lungs of A₃R^{-/-} mice treated with bleomycin, we measured the levels of eosinophil-related cytokine and chemokine transcripts in whole lung extracts (Figure 2D). Levels of CCL17 (TARC) and IL-5 were found to be elevated in the lungs of A₃R^{+/+} mice treated with bleomycin; however, their levels were not enhanced in the lungs of A₃R^{-/-} mice. However, transcript levels for CCL11 (eotaxin 1) and GM-CSF were significantly increased in the lungs of A₃R^{-/-} mice treated with bleomycin. Similar increases in protein levels of CCL11 were seen

Figure 2. Pulmonary eosinophil recruitment. (A) BAL was collected on Days 1, 3, 7, 14, and 21 after bleomycin exposure, and cells were cytopun onto slides, stained with Diff-Quick, and eosinophils counted using a hemocytometer. Triangles depict eosinophils from $A_3R^{+/+}$ mice exposed to bleomycin, while squares depict eosinophils from $A_3R^{-/-}$ mice exposed to bleomycin. Data are presented as mean cell counts $\times 10^4 \pm$ SEM. *Significant difference compared with $A_3R^{+/+}$ mice ($P < 0.05$, $n = 3-8$). (B) Lung sections from $A_3R^{+/+}$ and $A_3R^{-/-}$ mice 14 days after exposure to bleomycin were stained with mMBP-1 to mark eosinophils; scale bar = 100 μ m. (C) The number of mMBP-1-positive cells per mm^2 was quantified in lung sections using Image-Pro software. Data are presented as mean eosinophils/ $\text{mm}^2 \pm$ SEM. (D) Total cellular RNA was isolated from the lungs of wild-type ($A_3R^{+/+}$) or $A_3R^{-/-}$ (A_3R^{-}) mice 14 days after exposure to saline (open bars) or bleomycin (solid bars). Quantitative rtPCR was used to quantify the levels of key eosinophil related cytokines and chemokines. Values were normalized to 18S ribosomal RNA and are presented as mean normalized transcript levels (Δ^{-Ct}) $\pm$ SEM. *Significant difference from saline-treated $A_3R^{+/+}$ mice, #significant difference from bleomycin-treated $A_3R^{+/+}$ mice ($P < 0.05$, $n = 6-8$).

in the BAL fluid of these mice (Table E1). These results suggest that in the absence of A_3R signaling, there is increased production of key mediators that lead to the enhanced recruitment of eosinophils to the lung after bleomycin exposure.

The enhanced recruitment of eosinophils prompted us to examine eosinophil activation. There was a marked increase in EPO activity in the BAL fluid collected from $A_3R^{+/+}$ mice exposed to bleomycin (Figure 3A), suggesting substantial eosinophil activation. However, despite the increase in eosinophil numbers seen, there was no increase in EPO activity in the BAL fluid of $A_3R^{-/-}$ mice exposed to bleomycin. BAL cell pellets were disrupted and EPO activity monitored in homogenates to determine whether the lack of EPO activity in the BAL fluid of $A_3R^{-/-}$ mice was due to the absence of EPO protein in eosinophils. Results demonstrated that EPO activity was equally abundant in disrupted eosinophils from the airways of $A_3R^{+/+}$ and $A_3R^{-/-}$ mice exposed to bleomycin (Figure 3B), suggesting that the lack of EPO activity in the BAL of $A_3R^{-/-}$ mice is due to defects in eosinophil degranulation. These findings demonstrate that the eosinophil degranulation associated with bleomycin treatment is A_3R dependent.

Pulmonary Fibrosis in $A_3R^{-/-}$ Mice Treated with Bleomycin

$A_3R^{-/-}$ mice were exposed to bleomycin, and fibrosis endpoints were examined 14 days later to assess the contribution of A_3R signaling to pulmonary fibrosis. Masson's trichrome staining revealed extensive collagen deposition throughout the lungs of

$A_3R^{+/+}$ mice exposed to bleomycin (Figure 4A). Extensive collagen deposition was also seen in the lungs of $A_3R^{-/-}$ mice exposed to bleomycin. There were substantial increases in the levels of collagen transcripts and protein in the lungs of $A_3R^{+/+}$ mice challenged with bleomycin (Figures 4C and 4D). Collagen transcripts and protein levels were also elevated in the lungs of $A_3R^{-/-}$ mice exposed to bleomycin; however, the levels were slightly lower than those measured in $A_3R^{+/+}$ mice. Fibrosis was also assessed using α -smooth muscle actin (α -SMA) immunostaining for myofibroblasts. Results demonstrated a similar increase in α -SMA-positive myofibroblasts in the airways of $A_3R^{+/+}$ and $A_3R^{-/-}$ mice exposed to bleomycin (data not shown). Finally, Ashcroft scoring was used as a means of assessing the overall fibrosis seen. Consistent with our measurements of collagen, the degree of fibrosis in the lungs of $A_3R^{+/+}$ and $A_3R^{-/-}$ mice were equivalent (Figure 4B). Collectively, these results demonstrate that there is no substantial difference in the degree of bleomycin-induced pulmonary fibrosis in $A_3R^{+/+}$ and $A_3R^{-/-}$ mice.

TGF- β 1 Regulation in $A_3R^{-/-}$ Mice Treated with Bleomycin

Given the enhanced inflammation seen in the lungs of $A_3R^{-/-}$ mice exposed to bleomycin, it was surprising to find little difference in the degree of pulmonary fibrosis in these mice. The levels of the profibrotic mediator TGF- β 1 were examined to address the mechanisms involved in this discrepancy. As expected, levels of TGF- β 1 mRNA were elevated in lung extracts of $A_3R^{+/+}$ mice treated with bleomycin (Figure 5A). Interest-

Figure 3. Eosinophil activity. (A) Eosinophil peroxidase activity was measured in the BAL fluid of mice 14 days after exposure to saline or bleomycin. (B) Eosinophil peroxidase activity was measured in the BAL cell pellets from mice 14 days after exposure to saline or bleomycin. Eosinophil peroxidase activity is presented as mean optical density (OD) at 490 nm/ml $\pm$ SEM. *Significant difference from saline-treated A₃R^{+/+} mice, #significant difference from bleomycin-treated A₃R^{+/+} mice ($P < 0.05$, $n = 6-8$).

ingly, levels of TGF- β 1 mRNA were found to be hyperelevated in whole lung RNA extracts (Figure 5A) and RNA extracts from BAL cell pellets (Figure 5B) of A₃R^{-/-} mice treated with bleomycin. However, there was no difference in the levels of active TGF- β 1 protein in the BAL fluid of A₃R^{+/+} and A₃R^{-/-} mice exposed to bleomycin (Figure 5C). These findings suggest

that there is enhanced production of latent TGF- β 1 in the lungs of A₃R^{-/-} mice treated with bleomycin, but no increase in the active form of this mediator.

Studies have shown that MMP-9 is an important regulator of active TGF- β 1 production in pulmonary fibrosis (35). We therefore hypothesized that MMP-9 production is diminished in the

Figure 4. Pulmonary fibrosis in A₃R^{+/+} and A₃R^{-/-} mice. (A) A₃R^{+/+} and A₃R^{-/-} mice were killed 14 days after saline or bleomycin exposure, and the lungs were processed for Masson's trichrome staining. Photographs are representative of six to eight mice from each condition; scale bars = 100 μ m. (B) Ashcroft scores were determined by examining multiple sections from lungs of mice from each condition. Data are presented as mean Ashcroft score $\pm$ SEM ($n = 6-8$ lungs per condition). (C) RNA was extracted from the lungs of mice 14 days after saline or bleomycin exposure, and α 1 procollagen levels were quantified using quantitative rtPCR. Values were normalized to 18S ribosomal RNA and are presented as mean normalized transcript levels (Δ -Ct) $\pm$ SEM ($n = 6-8$). (D) Collagen protein levels were measured in whole lungs 14 days after saline or bleomycin exposure using the Sircol assay. Data are presented as mean μ g collagen per lung $\pm$ SEM ($n = 6-8$). *Significant difference from saline-treated A₃R^{+/+} mice ($P < 0.05$).

Figure 5. TGF- β 1 expression and activity. Total cellular RNA was isolated from the lungs (A) or BAL cell pellets (B) of wild-type ($A_3R^{+/+}$) or $A_3R^{-/-}$ mice 14 days after exposure to saline (open bars) or bleomycin (solid bars). Quantitative rtPCR was used to measure TGF- β 1 transcript levels. Values were normalized to 18S ribosomal RNA and are presented as mean normalized transcript levels (Δ^{-Ct}) $\pm$ SEM ($n = 6-8$). (C) Active TGF- β 1 protein levels were determined in BAL fluid from mice treated the same as in A and B. Values are presented as mean pg/ml TGF- β 1 $\pm$ SEM ($n = 4-6$). *Significant difference from saline-treated $A_3R^{+/+}$ mice, #significant difference from bleomycin-treated $A_3R^{+/+}$ mice ($P < 0.05$).

lungs of $A_3R^{-/-}$ mice, and this reduction may account for the lack of enhanced active TGF- β 1. To address this, MMP-9 levels were measured. MMP-9 whole lung mRNA levels (Figure 6A), whole lung protein activity (Figure 6B), and BAL fluid activity (Figure 6C) were all elevated in the lungs of $A_3R^{+/+}$ mice treated with bleomycin. Levels of MMP-9 RNA and activity were diminished in the lungs of $A_3R^{-/-}$ mice treated with bleomycin, suggesting that the A_3R regulates the production of this protease. MMP-9 activity is also regulated by the levels of protease inhibitors such as TIMP-1. TIMP-1 protein levels were found to be elevated in the BAL fluid of $A_3R^{+/+}$ mice treated with bleomycin, while levels were significantly reduced in the BAL fluid of $A_3R^{-/-}$ mice (Figure 6D). Consistent with these findings, there was a reduction in the ratio of MMP-9 to TIMP-1 activity in the BAL fluid of $A_3R^{-/-}$ mice (Figure 6E), further indicating a reduction in MMP-9 activity.

Examination of the cellular source of MMP-9 in this model using immune detection methods revealed that MMP-9 production is limited to inflammatory components in the lungs of both $A_3R^{+/+}$ and $A_3R^{-/-}$ mice treated with bleomycin (Figures 7A and 7B). Examination of isolated BAL cells revealed that all

neutrophils and a subset of alveolar macrophages produced MMP-9 in the lungs of bleomycin-treated $A_3R^{+/+}$ mice (Figure 7C). In contrast, MMP-9 production was detected in neutrophils isolated from the airways of $A_3R^{-/-}$ mice treated with bleomycin; however, alveolar macrophages isolated from these mice did not produce MMP-9 (Figure 7D). MMP-9 expression was not detected in eosinophils in either group of mice (data not shown). MMP-9 expression was abundant in mast cells found in the bronchi of $A_3R^{+/+}$ mice treated with bleomycin (Figures 7E and 7F), but was absent from mast cells in the bronchi of $A_3R^{-/-}$ mice (Figures 7G and 7H). Collectively, these findings demonstrate that MMP-9 levels are diminished in the lungs of $A_3R^{-/-}$ mice and that A_3R -dependent expression of MMP-9 in mast cells and alveolar macrophages likely accounts for this reduction.

DISCUSSION

Regulated inflammation and matrix production are critical components of wound healing processes. An emerging model for the development of pulmonary fibrosis is that abnormal

Figure 6. Matrix metalloproteinase (MMP)-9 and tissue inhibitor of metalloproteinase (TIMP)-1 expression and activity. (A) Total cellular RNA was isolated from the lungs of wild-type ($A_3R^{+/+}$) or $A_3R^{-/-}$ mice 14 days after exposure to saline (open bars) or bleomycin (solid bars). Quantitative rtPCR was used to quantify the levels MMP-9 transcripts. Values were normalized to 18S ribosomal RNA and are presented as mean normalized transcript levels (Δ^{-Ct}) $\pm$ SEM ($n = 6-8$). (B) BAL fluid (20 μ l from saline-, and 10 and 5 μ l from bleomycin-treated samples) were subjected to 10% SDS-PAGE in gels containing 0.02% gelatin. Gels were fixed and stained with 50% methanol and 10% acetic acid that contained 0.3% wt/vol Coomassie blue. MMP-9 and MMP-2 bands were identified by their molecular weight. MMP-9 (C) and TIMP-1 (D) activities were determined in BAL fluid using

specific immunoassays. (E) MMP-9 and TIMP-1 BAL fluid activities were used to determine a ratio of MMP-9 to TIMP-1. Data are presented as means $\pm$ SEM. *Significant difference from saline-treated $A_3R^{+/+}$ mice, #significant difference from bleomycin-treated $A_3R^{+/+}$ mice ($P < 0.05$, $n = 6-8$).

Figure 7. MMP-9 immunolocalization. Mice were killed 14 days after saline or bleomycin exposure, and the lungs were lavaged and processed for MMP-9 immunolocalization. Immunoperoxidase detection using a MMP-9 antibody was used to localize MMP-9 (brown stain) to inflammatory cells in the lungs of (A) A₃R^{+/+} mice exposed to bleomycin and (B) A₃R^{-/-} mice exposed to bleomycin. The same antibody was used in an immunofluorescence assay to examine MMP-9 expression (green) in BAL cells from (C) A₃R^{+/+} mice exposed to bleomycin and (D) A₃R^{-/-} mice exposed to bleomycin. Blue staining denotes DAPI-stained nuclei. Mast cells were localized in bronchi of (E) A₃R^{+/+} mice exposed to bleomycin and (G) A₃R^{-/-} mice exposed to bleomycin using toluidine blue staining. MMP-9 immunoperoxidase detection (brown stain) was used to localize MMP-9 to mast cells in the bronchi of (F) A₃R^{+/+} mice exposed to bleomycin and (H) A₃R^{-/-} mice exposed to bleomycin. n, neutrophils; Φ , macrophages; arrows denote mast cells. Scale bars: A and B, 50 μ m; C and D, 10 μ m; H, 10 μ m (also applies to E-G).

inflammation and matrix regulation after lung injury progresses to an overactive wound healing response that culminates in fibrosis. In this context, understanding the factors produced after injury and how they influence the normal or abnormal wound healing response could provide important mechanistic information. Adenosine is produced in response to tissue damage, where it is thought to regulate key features of the injury response. Antiinflammatory and tissue-protective activities of adenosine are well documented (17, 18, 36), and adenosine signaling pathways can promote wound healing (13). In addition, recent studies demonstrate that adenosine has profibrotic activities in disease models including the lung (14, 15, 37). Thus, adenosine production after lung injury may access pathways that regulate the balance of inflammation and matrix production that influence lung repair or the progression to fibrosis. Adenosine elicits many of its activities by engaging cell surface adenosine receptors (12). The goal of this study was to examine the contribution of the A₃R to inflammatory and fibrotic processes after injury in the lung. This was done by subjecting A₃R^{-/-} mice to bleomycin-induced pulmonary injury. The results of these studies demonstrate that A₃R signaling serves a largely antiinflammatory role in the bleomycin model; however, it also serves to increase the levels of MMP-9 that may promote TGF- β 1 activation and downstream fibrotic pathways. These findings suggest that A₃R signaling may be an important pathway linking inflammation to the progression of pulmonary fibrosis.

A major finding of this study was that mice deficient in the A₃R develop enhanced pulmonary inflammation after bleomycin exposure. This was characterized by an increase in the numbers of macrophages, neutrophils, and particularly eosinophils in A₃R^{-/-} mice exposed to bleomycin. These findings suggest that the A₃R plays an antiinflammatory role in the bleomycin model. Similar

findings were reported in a study that demonstrated increased inflammation in A₃R^{-/-} mice subjected to an air-pouch model of acute inflammation (38). In addition, our observations are consistent with findings in *in vivo* models of injury, including lipopolysaccharide-induced endotoxemia (39), adjuvant-induced arthritis (40), colitis (41), and ischemic injury in the lung (42), where A₃R activation has been shown to suppress inflammatory processes. Thus, our findings strengthen the notion that the A₃R plays an important role in regulating the degree of inflammation after injury.

We also demonstrate for the first time that the A₃R is required for the release of EPO from eosinophils recruited to the lung. Despite the enhanced numbers of eosinophils recruited to the lungs of A₃R^{-/-} after bleomycin exposure, there was no evidence of EPO release from these cells, leading us to conclude that the A₃R is needed for eosinophil degranulation. A₃R expression is abundant on mouse (21) and human eosinophils (43), and engagement of this receptor on eosinophils increases intracellular Ca²⁺ levels (43). However, the role of the A₃R in eosinophil degranulation is controversial. *In vitro* studies have demonstrated that engagement of the A₃R on guinea pig pulmonary eosinophils can induce superoxide production (26). In contrast, studies on cultured human peripheral blood eosinophils do not support a role for A₃R engagement in stimulating eosinophil degranulation (43, 44), and there is evidence that it may even prevent degranulation (25). These discrepancies may arise in part from the inability of the *in vitro* setting to accurately capture the environment of the diseased lung where degranulation occurs. In support of this, we isolated peripheral blood eosinophils as well as allergen-recruited BAL eosinophils from transgenic mice overexpressing IL-5 (45), and we were not able to directly stimulate EPO release *in vitro* with an A₃R agonist (data not shown). We

thus conclude that important co-stimulators or cellular interactions necessary for A₃R-mediated eosinophil degranulation are not present in culture situations. A role for the A₃R in eosinophil degranulation *in vivo* may have important implications for diseases such as asthma, in which both elevations in eosinophils and adenosine are thought to be involved in disease progression (5, 46). Indeed, a recent expression study assessing transcripts from the lungs of allergen-treated wild-type and eosinophil-less mice demonstrated that A₃R transcripts were significantly down-regulated in the lungs of mice lacking eosinophils (47).

In the bleomycin model, pulmonary inflammation peaks between Days 7 and 10 after exposure and then diminishes, while fibrotic changes appear around Day 7 and progressively increase (48). Work from our laboratory and others has demonstrated that perturbations that lead to enhanced inflammation in this model are associated with enhanced fibrosis (11, 49, 50). Interestingly, we found that despite enhanced pulmonary inflammation in A₃R^{-/-} mice exposed to bleomycin, there was not an increase in standard measures of pulmonary fibrosis, including collagen production and deposition and α -SMA staining. This observation suggests that removing the A₃R uncouples amplification pathways that link inflammation and the severity of fibrosis. One mechanism that may be responsible for this is the regulation of MMP-9 expression. This protease is known to be elevated in fibrotic lungs diseases (51) and can contribute to fibrosis by promoting the processing of latent TGF- β 1 into active TGF- β 1 (35, 52). Our results demonstrate that MMP-9 expression is decreased in the lungs of A₃R^{-/-} mice exposed to bleomycin and that loss of expression in alveolar macrophages and mast cells is responsible for this decrease. In addition, we found that TGF- β 1 expression levels were elevated in A₃R^{-/-} mice treated with bleomycin; however, there was not a concomitant increase in the levels of active TGF- β 1. These findings have led us to propose a model in which adenosine generated during bleomycin injury (11) can engage the A₃R on alveolar macrophages and mast cells to promote the production of MMP-9 that in turn can lead to the activation of TGF- β 1 and contribute to the amplification of pulmonary fibrosis. This mechanism could account for the observation that despite increased inflammation in A₃R^{-/-} mice exposed to bleomycin, there is not an increase in the degree of fibrosis seen. This provides a novel route by which a factor generated in response to damage (namely, adenosine) can impact inflammation and subsequent fibrosis.

Another consideration for the disconnection of inflammation and fibrosis is the lack of eosinophil degranulation. Eosinophils are present in the lungs of patients with idiopathic lung fibrosis and are elevated in models of bleomycin-induced fibrosis (53, 54). Moreover, eosinophil-derived cationic proteins such as EPO have been shown to influence the production of mediators such as MMP-9 and TGF- β 1 from pulmonary cells (55), suggesting that eosinophil degranulation may play an important role in regulating fibrosis. Although we found increased numbers of eosinophils in the lungs of A₃R^{-/-} mice exposed to bleomycin, they did not appear to be degranulating as evidenced by the lack of EPO release. Thus, the lack of release of eosinophil granule proteins may contribute to the lack of enhanced fibrosis in A₃R^{-/-} mice exposed to bleomycin.

In conclusion, this study has characterized the contribution of the A₃R in the inflammation and fibrosis that results from bleomycin injury. The roles of this receptor in this model are complex. There was clear evidence for antiinflammatory influences in that A₃R^{-/-} mice exhibited exaggerated expression of certain cytokines and chemokines in association with an excessive influx of multiple inflammatory cell types. There was essentially no difference in the degree of fibrosis seen in A₃R^{+/+} and A₃R^{-/-} exposed to bleomycin, suggesting that the A₃R is not necessary for the development of fibrosis in this model. However, investigation of TGF- β 1 activation suggests that the A₃R may contribute to the

amplification of pulmonary fibrosis by regulating MMP-9, and/or by regulating eosinophil activation. Thus, signaling through the A₃R may contribute to inflammatory and fibrotic processes that regulate the balance between normal wound healing and the unremitting fibrosis seen in interstitial lung diseases.

Conflict of Interest Statement: M.R.B. is a paid consultant for CV Therapeutics for work on the A_{2B} adenosine receptor. None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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