Adenosine-Dependent Pulmonary Fibrosis in Adenosine Deaminase-Deficient Mice

Janci L. Chunn, Jose G. Molina, Tiejuan Mi, Yang Xia, Rodney E. Kellems and Michael R. Blackburn

*J Immunol* 2005; 175:1937-1946; doi: 10.4049/jimmunol.175.3.1937

http://www.jimmunol.org/content/175/3/1937

---

**Why The JI?**

- **Rapid Reviews! 30 days*** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

---

**References**

This article **cites 66 articles**, 20 of which you can access for free at:
http://www.jimmunol.org/content/175/3/1937.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

---

*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2005 by The American Association of Immunologists All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Adenosine-Dependent Pulmonary Fibrosis in Adenosine Deaminase-Deficient Mice

Janci L. Chunn, Jose G. Molina, Tiejuan Mi, Yang Xia, Rodney E. Kellems, and Michael R. Blackburn

Pulmonary fibrosis is a common feature of numerous lung disorders, including interstitial lung diseases, asthma, and chronic obstructive pulmonary disease. Despite the prevalence of pulmonary fibrosis, the molecular mechanisms governing inflammatory and fibroproliferative aspects of the disorder are not clear. Adenosine is a purine-signaling nucleoside that is generated in excess during cellular stress and damage. This signaling molecule has been implicated in the regulation of features of chronic lung disease; however, the impact of adenosine on pulmonary fibrosis is not well understood. The goal of this study was to explore the impact of endogenous adenosine elevations on pulmonary fibrosis. To accomplish this, adenosine deaminase (ADA)-deficient mice were treated with various levels of ADA enzyme replacement therapy to regulate endogenous adenosine levels in the lung. Maintaining ADA-deficient mice on low dosages of ADA enzyme therapy led to chronic elevations in lung adenosine levels that were associated with pulmonary inflammation, expression of profibrotic molecules, collagen deposition, and extreme alteration in airway structure. These features could be blocked by preventing elevations in lung adenosine. Furthermore, lowering lung adenosine levels after the establishment of pulmonary fibrosis resulted in a resolution of fibrosis. These findings demonstrate that chronic adenosine elevations are associated with pulmonary fibrosis in ADA-deficient mice and suggest that the adenosine functions as a profibrotic signal in the lung.

Received for publication January 26, 2005. Accepted for publication May 19, 2005.

Copyright © 2005 by The American Association of Immunologists, Inc. 0022-1767/05/$02.00
FIGURE 1. Modulation of lung adenosine levels using ADA enzyme therapy. Mice were administered on weekly injections of PEG-ADA according to the low/high-dose regimens outlined in the Materials and Methods, where LD-ADA denotes low-dose regimen and HD-ADA denotes high-dose regimen. Mice were sacrificed at 16 wk of age, and lung adenosine levels were measured using reversed-phase HPLC. Values are given as mean nanomoles of adenosine per milligram of protein ± SEM (n = 8 samples for each group). *, Significant elevation in adenosine levels over the other groups; #, significant reduction in adenosine levels from \( \text{ADA}^{-/-} \)-LD-ADA group; p < 0.05 using a Student’s t test.

long-term consequences of adenosine elevations on pulmonary injury. In the current study, exogenous ADA enzyme therapy was used to extend the life span of these mice, allowing for investigation of the impact of chronic elevations in lung adenosine on pulmonary disease. In this model, adenosine elevations were associated with pulmonary inflammation and excessive pulmonary fibrosis. This fibrosis could be blocked by preventing the elevations in lung adenosine, suggesting that altered adenosine signaling was causative. Furthermore, lowering lung adenosine levels after the establishment of pulmonary fibrosis resulted in a resolution of fibrosis in the lung, suggesting that adenosine signaling plays an essential role in the regulation of profibrotic pathways.

Materials and Methods

Mice

ADA-deficient mice were generated and genotyped as described previously (25). Mice homozygous for the null \( \text{Ada}^{-/-} \) allele were designated ADA-deficient (\( \text{ADA}^{-/-} \)), whereas mice heterozygous for the null \( \text{Ada} \) allele were designated as ADA control mice (\( \text{ADA}^+ \)). All mice were on a 129sv/C57BL/6j mixed background, and all phenotype comparisons were performed among littermates. Animal care was in accordance with institutional and National Institutes of Health guidelines. All mice were housed in ventilated cages equipped with microisolator 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.

ADA enzyme therapy

Polyethylene glycol-conjugated ADA (PEG-ADA) was prepared as described previously (26). \( \text{ADA}^{-/-} \) mice received i.m. injections of PEG-ADA on postnatal days 1, 5, 9, 13, and 17 (0.625, 1.25, 2.5, 2.5, and 2.5 U, respectively) (1 U is defined as the amount necessary to convert 1 \( \mu \text{M} \) of adenosine to inosine/min at 25°C). Mice were then injected i.p. with 5 U of PEG-ADA on a weekly basis until 1 mo of age. At 1 mo of age, a group of mice were tapered down to a low dose of PEG-ADA using the following weekly i.p. injection schedule (0.625 U for 1 wk, 0.25 U for 4 wk, and then 0.125 U for 4–7 wk). This dosing protocol was designated a “low-dose” ADA treatment regimen (LD-ADA). Other mice were maintained on a weekly 5-U i.p. dose of PEG-ADA for the duration of the experimental period (16 wk). This dosing protocol was designated a “high-dose” ADA treatment regimen (HD-ADA). To examine the impact of ADA enzyme therapy on established pulmonary end points, mice were treated according to the LD-ADA regimen described above for 13 wk and were then given weekly i.p. injections of 5 U of PEG-ADA for 5 wk. For all experiments, \( \text{ADA}^+ \) mice and \( \text{ADA}^{-/-} \) mice treated with HD-ADA were used as controls.
Quantification of lung adenosine levels

Mice were anesthetized, and the lungs were removed rapidly and frozen in liquid nitrogen. Adenosine nucleosides were extracted from frozen lungs using 0.4 N perchloric acid as previously described (27), and adenosine was separated and quantified using reversed-phase HPLC.

Histological analysis

Mice were anesthetized, and the lungs were perfused with 5–10 ml of PBS and then infused with 0.5 ml of fixative (4% paraformaldehyde in PBS) and fixed overnight at 4°C. Fixed lungs were rinsed in PBS, dehydrated through graded ethanol washes, and embedded in paraffin. Sections (5 μm) were collected on slides and stained with H&E or Masson’s trichrome, according to the manufacturer’s instructions.

Collagen quantification

The Sircol collagen assay (Biocolor) was performed on snap frozen whole lungs. Lungs were homogenized in 5 ml of 0.5 M acetic acid with 20 mg of pepsin and incubated with shaking for 24 h at 4°C. Homogenate was incubated with the primary Ab at a 5-fold greater concentration before incubation. Active and total TGF-β1 levels were assayed in BAL fluid using a kit from R&D Systems.

Analysis of mRNA

Mice were anesthetized, and the lungs were rapidly removed and frozen in liquid nitrogen. RNA was isolated from frozen lung tissue using TRIzol Reagent (Invitrogen Life Technologies). RNA samples were then DNase treated and subjected to quantitative real-time RT-PCR. The primers, probes, and procedures for real-time RT-PCR for the ARs and IL-13 were described previously (29, 30). The primers and TaqMan probe for mouse, r1-procollagen were as follows: forward, 5′-GGTGAACTGTGGTGACCT-3′, and reverse, 5′-CTCTTACCAAGGAGAACCACATC-3′, and probe, 5′-FAM ACAGAGGTTGCTGCTGTCGAGGTCGGG-3′. Reactions were conducted on a Smart Cycler rapid thermal cycler system (Cepheid). Specific transcript levels were determined using Smart Cycler analysis software through comparison to a standard curve generated from the PCR amplification of template dilutions.

Results

Modulation of lung adenosine levels using ADA enzyme therapy

A primary function of ADA is to control the levels of adenosine in tissues and cells. ADA−/− mice exhibit increases in lung adenosine levels in conjunction with extensive pulmonary inflammation, injury, and death by 3 wk of age (25). The rapid onset of pulmonary inflammation and death prevented the examination of the effects of chronic elevations in adenosine. To establish a model system to investigate the consequences of chronic adenosine elevations, ADA−/− mice were treated with varying doses of exogenous PEG-ADA over a period of 16 wk, and lungs were collected for adenosine quantification (Fig. 1). ADA−/− mice on LD-ADA showed marked increases in lung adenosine levels as compared with ADA−/− mice, whereas ADA−/− mice kept on HD-ADA did not develop significant elevations in lung adenosine. These findings substantiate that continuous HD-ADA enzyme therapy can effectively prevent elevations in lung adenosine in ADA−/− mice, while LD-ADA allows elevations in lung adenosine.

ADA−/− mice treated with chronic elevations in lung adenosine exhibit extensive lung collagen deposition and α-smooth muscle expression

ADA−/− mice treated with LD-ADA showed external features of respiratory distress, including rapid, labored breathing, rib cage...

**FIGURE 3.** Airway cellularity following ADA enzyme therapy. Cellularity was determined in the lungs of 16-wk-old mice treated with LD-ADA or HD-ADA. A, Total cell numbers obtained from BAL fluid. B, BAL cells were collected, cytospun onto slides, and stained with Diff-Quick for determination of cellular differentials. Data are presented as mean cell counts ± SEM (n = 5); *, significant elevation over the other groups; #, significant reduction in total cells from ADA−/− mice treated with LD-ADA (p < 0.05).

α-Smooth muscle actin (sma) and TGF-β1 immunohistochemistry

Immunohistochemistry was performed on 5-μm sections cut from formalin-fixed, paraffin-embedded lungs. Sections were rehydrated through graded ethanol washes, and embedded in paraffin. Sections (5 μm) were collected on slides and stained with H&E or Masson’s trichrome, according to the manufacturer’s instructions.
deformities, and pulmonary crackles. Histological analysis showed that mice treated with LD-ADA had extensive pulmonary remodeling, including alveolar epithelial cell hyperplasia and hypertrophy, smooth muscle cell thickening and alveolar invasion, severe pulmonary inflammation, and apparent fibrosis and matrix deposition (data not shown). To more accurately characterize the fibrotic phenotype seen, various end points associated with fibrosis were examined. Collagen deposition was found to be increased in the lungs of ADA−/− mice treated with LD-ADA as detected by intense blue staining with Masson’s trichrome (Fig. 2B). These findings were confirmed by demonstrating that the production of α1-procollagen transcripts (Fig. 2G) and total lung collagen content (Fig. 2H) were also markedly increased in the lungs of ADA−/− mice treated with LD-ADA. In addition to collagen deposition, extensive α-smooth muscle actin immunostaining was detected in the lungs of ADA−/− mice treated with LD-ADA (Fig. 2E), suggesting increased numbers of myofibroblasts. Collagen production and deposition as well as increases in myofibroblasts were prevented in ADA−/− mice given HD-ADA therapy (Fig. 2, C, F, G, and H). These alterations in fibrosis were also associated with overall changes in Ashcroft fibrotic scores (Fig. 2I). Taken together, these data suggest a correlation between elevations in lung adenosine and the elaboration of a fibrotic phenotype within the lung.

**ADA−/− mice develop severe pulmonary inflammation**

Pulmonary fibrosis is often associated with an inflammatory cell component that is predominantly comprised of alveolar macrophages (2). Histological analysis of the lungs of ADA−/− mice following the LD-ADA regimen revealed extensive pulmonary inflammation within remodeled alveoli as well as the pulmonary interstitium (data not shown). To characterize this inflammation, total cell numbers and cellular differentials were assessed in recovered BAL fluid. There was a 9-fold increase in total cell numbers obtained from the BAL fluid of ADA−/− mice following LD-ADA treatment, as compared with untreated ADA+ mice and ADA+ mice following HD-ADA treatment (Fig. 3A). Furthermore, ADA−/− mice treated with HD-ADA exhibited normal levels of total cells recovered from the BAL fluid (Fig. 3A). Cellular differentials from ADA−/− mice treated with LD-ADA showed a 6-fold increase in alveolar macrophages and a significant elevation in lymphocytes, eosinophils, and neutrophils (Fig. 3B). These increases were not seen in ADA−/− mice treated with HD-ADA. In addition, the macrophages obtained from ADA−/− mice treated with LD-ADA were large and foamy, indicative of an activated state. This was not apparent in the other groups where the macrophages appeared quiescent. Taken together, these results suggest that chronic elevations in lung adenosine promote inflammatory cell infiltration into the lung.

**Profibrotic mediators are elevated in the lungs of ADA−/− mice treated with LD-ADA**

IL-1β (31), IL-13 (32), Pai-1 (33), OPN (34), and matrix metalloproteinase (MMP)-2 (35) are profibrotic mediators that have been correlated with pulmonary fibrosis. Transcript levels of these mediators were assessed in the lungs of ADA−/− mice treated with LD- or HD-ADA to determine whether profibrotic mediators were increased in the lungs of ADA−/− mice treated with LD-ADA (Fig. 4, A–E). These increases were prevented in ADA−/− mice.
The findings described above demonstrate that chronic elevations in lung adenosine correlate with pulmonary fibrosis in the lungs of ADA−/− mice. To determine whether ADA enzyme therapy could lower lung adenosine levels after damage had occurred, ADA−/− mice were treated with the LD-ADA regimen for 13 wk and then brought up to a HD-ADA treatment regimen for 5 wk. Analysis of lung adenosine levels revealed that this LS-HD-ADA treatment was able to return lung adenosine levels to control values (Fig. 6). These data demonstrate that ADA therapy can reverse lung adenosine elevations in ADA−/− mice with established lung disease.

**FIGURE 5.** TGF-β1 levels in ADA−/− mice treated with ADA enzyme therapy. Immunostaining for TGF-β1 was determined in the lungs of 16-wk-old mice treated with LD-ADA or HD-ADA. A, ADA+/+ mice; B, ADA−/− on a LD-ADA regimen; and C, ADA−/− mice on a HD-ADA regimen. D, ADA−/− lung section incubated with peptide control for Ab specificity. Findings are representative of at least five mice per group. Scale bars = 100 μm. E, Quantitative RT-QPCR analysis for transcripts of TGF-β1 in whole lung RNA extracts of 16-wk-old mice treated with LD-ADA or HD-ADA. Data are presented as mean transcripts per picogram RNA ± SEM (n = 4). F, Total TGF-β1 protein levels were determined in the BAL fluid of 16-wk-old mice treated with LD-ADA or HD-ADA. Data are presented as mean pg/ml ± SEM (n = 4–6 in each group). G, Active TGF-β1 protein levels were determined in the BAL fluid of 16-wk-old mice treated with LD-ADA or HD-ADA. Data are presented as mean pg/ml ± SEM (n = 4–6 in each group). *, Significant elevations in transcripts over controls; #, significant reduction from ADA−/− mice treated with LD-ADA (p < 0.05).

Reversal of lung adenosine levels in ADA−/− mice receiving “late-stage” (LS)-HD-ADA enzyme therapy

The findings described above demonstrate that chronic elevations in lung adenosine levels were able to return lung adenosine levels to control values (Fig. 6). These data demonstrate that ADA therapy can reverse lung adenosine elevations in ADA−/− mice with established lung disease.
FIGURE 7. Normalization of lung collagen and α-smooth muscle actin (α-sma) expression following ADA enzyme therapy. Lung sections were stained with Masson’s trichrome. A, Lung from untreated ADA−/− mouse; B, lung from ADA−/− mouse treated with LD-ADA until 13 wk of age; C, ADA−/− mouse treated with LD-ADA until 13 wk of age and then treated with HD-ADA for 5 wk (LS-HD-ADA). Insets represent α-sma immunohistochemistry from the respective samples. Data represent findings from at least six mice per group. Scale bar = 100 μm in figures and 10 μm in insets. D, Quantitative RT-PCR for α1-procollagen in whole lung RNA extracts from mice treated as described in A–C. Data are presented as mean α1-procollagen transcripts per picogram RNA ± SEM (n = 6). E, Soluble collagen protein levels in BAL fluid (n = 4–8 samples/group). F, Mean Ashcroft scores ± SEM (n = 4–8 samples/group). *, Significant elevation over all groups; #, significant reduction from ADA−/− LD-ADA (p < 0.05).

Lung inflammation is reduced in ADA−/− mice following ADA treatment

To determine whether lowering lung adenosine levels could alter the inflammatory status of ADA−/− mice, BAL cellularity was examined. There was a 5-fold increase in total cells recovered from BAL fluid of ADA−/− mice on LD-ADA regimens with HD-ADA for 5 wk (LS-HD-ADA) resulting in a significant decrease in lung collagen production (Fig. 7C). Treatment of ADA−/− mice on LD-ADA regimens with HD-ADA for 5 wk (LS-HD-ADA) resulted in a significant decrease in lung collagen production (Fig. 7C). Lung inflammation following ADA treatment can halt and reverse pulmonary fibrosis and implies a correlation between the modulation of lung adenosine levels and the elaboration of collagen deposition in the lungs of ADA−/− mice.

Profibrotic mediators are reduced in ADA−/− mice following ADA treatment

To investigate whether lowering lung adenosine levels would alter transcript levels of pro-fibrotic mediators, lung transcripts for IL-1β, Pai-1, OPN, and MMP-2 were assessed in ADA−/− mice following LS-HD-ADA treatment. Results showed that levels of profibrotic mediators were reduced in ADA−/− mice following LS-HD-ADA treatment (Fig. 8A–D). Similarly, the enhanced levels of profibrotic mediators following LS-HD-ADA treatment revealed a reduction in macrophages, lymphocytes, eosinophils, and neutrophils (Fig. 8B). These results demonstrate that ADA treatment can improve pulmonary inflammation in ADA−/− mice.

FIGURE 8. Normalization of airway inflammation following ADA enzyme therapy. A, Total cell numbers obtained from BAL fluid of ADA−/− mice treated with LD-ADA for 13 wk or ADA−/− mice treated with LD-ADA for 13 wk and then HD-ADA for 5 wk (LS-HD-ADA). B, Cellular differentials of BAL cells collected from mice described in A. Values are presented as mean total cells ± SEM (n = 5); *, significant elevation over ADA−/−; #, significant reduction from ADA−/− LD-ADA (p < 0.05).
of TGF-β1 seen in the lungs of \( ADA^{-/-} \) mice on LD-ADA treatment for 13 wk (Fig. 10, A and C–E) were lowered following HD-ADA treatment for 5 wk (Fig. 10, B and C–E). These results demonstrate that exogenous ADA can lower profibrotic mediator production in the lungs of \( ADA^{-/-} \) mice.

AR transcripts are altered in the lungs of \( ADA^{-/-} \) mice following ADA enzyme therapy

AR levels are known to be altered in chronic lung diseases where adenosine levels are elevated (24). To investigate the levels of various ARs in the lungs of \( ADA^{-/-} \) mice exhibiting pulmonary fibrosis, AR transcript levels were quantified in whole lung extracts from \( ADA^{-/-} \) mice treated with various dosages of PEG-ADA (Fig. 11). There was a significant elevation in transcript levels for the A1AR, A2AAR, and A3AR in the lungs of \( ADA^{-/-} \) mice on a LD-ADA treatment regimen. These elevations were prevented with HD-ADA therapy. There were no significant changes in the transcript levels of the A2BAR in any of the treatment groups. LS-HD-ADA treatment lowered A2AAR transcript levels to control values but did not significantly alter the transcript levels of the A1AR, A2BAR, or A3AR in the lungs of \( ADA^{-/-} \) mice. These findings demonstrate that AR levels are altered in the lungs of \( ADA^{-/-} \) mice exhibiting pulmonary fibrosis.

Discussion

Adenosine has been implicated in the regulation of chronic lung disease; however, there are few studies that have examined the contributions of endogenous adenosine to specific cellular processes within the inflamed lung. In the current study, ADA enzyme replacement therapy was used to regulate the levels of endogenous adenosine in \( ADA^{-/-} \) mice. This approach demonstrated that chronic elevations in lung adenosine levels led to the development of severe pulmonary fibrosis. Preventing or reversing elevations in adenosine levels in this model was associated with prevention or...
reversal of pulmonary fibrosis, suggesting that elevations in lung adenosine were causative in the resulting condition.

IPF is a particularly severe form of pulmonary fibrosis in humans (2). It is progressive in nature, does not respond well to corticosteroid treatments, and usually leads to death within 5 years. The poor response to corticosteroid treatment has led to the hypothesis that it is not unremitting pulmonary inflammation that leads to the progression of fibrosis in IPF but rather structural changes in the lung architecture (36). Structural abnormalities of the lung appear to result from pathways that mediate airway epithelial cell integrity (37), extracellular matrix turnover (38), and myofibroblast recruitment and maintenance (39). Understanding the complex regulation of these processes in IPF has been difficult due to the lack of model systems that exhibit features of progressive pulmonary fibrosis. The progressive nature of the pulmonary fibrosis seen in ADA−/− mice suggests that adenosine may access pathways that maintain or promote the progression of pulmonary fibrosis. This is supported by the observation that lowering adenosine levels during the progression of pulmonary fibrosis can halt and even reverse aspects of pulmonary inflammation and fibrosis in ADA−/− mice. This model will be useful in examining mechanisms by which adenosine mediates fibrosis, as well as investigating the cellular signaling pathways involved in the maintenance, progression, and resolution of pulmonary fibrosis.

A key finding of this study was the adenosine-dependent elevation of key regulators of pulmonary inflammation and fibrosis in the lungs of ADA−/− mice. Numerous cellular mediators have been implicated in the ontogeny and maintenance of pulmonary fibrosis (2, 36, 40). Among these, IL-1β and IL-13 are important proinflammatory signals (31, 32), whereas TGF-β1, Pai-1, OPN, and MMP-2 are involved in the regulation of the extracellular matrix environment and structural integrity of the airways (41–44). The levels of all these mediators are elevated in patients and animal models exhibiting pulmonary fibrosis (35, 45–48), and studies in transgenic and knockout mice demonstrate their importance in bleomycin-induced pulmonary fibrosis (34, 42, 49). The current study demonstrates that these mediators are all substantially elevated in the lungs of ADA−/− mice that exhibit chronic elevations of adenosine and fibrosis. Moreover, lowering levels of endogenous adenosine in ADA−/− mice with established pulmonary fibrosis was able to reverse elevations of these mediators. Although these findings suggest that adenosine elevations lead to the activation of pathways classically associated with fibrosis, they do not distinguish which of these mediators are directly regulated by adenosine in this model. Along these lines, the regulation of TGF-β1 and IL-13 are of particular interest because they have been shown to be sufficient for the induction of fibrotic pathways such as is seen in the lungs of ADA−/− mice (32, 50). Furthermore, IL-13 has been implicated in an adenosine amplification pathway in the lungs of ADA−/− mice (51), and TGF-β1 and IL-13 are themselves in parallel pathways (50). Current efforts are underway to determine the ability of adenosine to directly regulate these and other profibrotic mediators, as well as to determine the relative contribution of these mediators to the pulmonary fibrosis seen in ADA−/− mice. These studies will provide important information into the mechanisms by which adenosine regulates fibrosis in the lung.

Many of the cellular processes that have been implicated in pulmonary inflammation and airway damage have been directly or indirectly associated with adenosine signaling (52, 53). Adenosine elicits many of its actions by engaging cell surface ARs. Four ARs have been characterized, each with a unique affinity for adenosine and unique connections to cellular signaling components via various G proteins (12). Engagement of ARs can promote aspects of inflammation and remodeling, including the induction of apoptosis (54), the promotion of cell survival or differentiation (55), cytokine and chemokine production (29, 56–61), extracellular matrix deposition (62), and protease activation (30, 63). Thus, it is reasonable to suggest that adenosine generated in the inflamed and damaged lung may contribute to various cellular processes in the fibrotic program. Perhaps the most compelling evidence that adenosine can directly activate profibrotic pathways is a recent study in human pulmonary fibroblasts cultured ex vivo, where adenosine was shown to promote the differentiation of pulmonary fibroblasts into collagen-producing myofibroblasts (21). This response involved the engagement of the A2B AR and the release of IL-6, and these responses were enhanced in the presence of hypoxia. The transformation of pulmonary fibroblasts into myofibroblasts is considered a major mechanism underlying pulmonary fibrosis (36, 39).
The demonstration that adenosine can directly promote this process further strengthens the likelihood that this nucleoside is in itself profibrotic. It appears that engagement of the A2B AR may be an important mechanism by which adenosine promotes pulmonary fibrosis (21). This would imply that treatment with selective A2B AR antagonist may have some use in regulating pulmonary fibrosis. In the current study, we demonstrate that there are increased numbers of myofibroblasts and collagen deposition in the lungs of ADA−/− mice and that all of the ARs are expressed in this environment. Interestingly, A2B AR was the most abundant of the four ARs in the lung. Also of interest was the observation that AR levels changed with the degree of inflammation and fibrosis seen. The significance of these changes is not yet clear, but they provide evidence for the existence of adenosine signaling pathways in these mice. Examination of the AR functions in the lungs of ADA−/− mice using AR antagonists and knockout mice will help to define the functions of individual ARs in adenosine-dependent pulmonary fibrosis.

Collagen deposition by pulmonary myofibroblasts is a major feature of pulmonary fibrosis (2, 36, 39). Much of the myofibroblast proliferation and collagen deposition seen in the lungs of ADA−/− mice maintained on LD-ADA was located in the distal alveolar airways. However, collagen deposition was also noted around bronchial airways (data not shown); a feature of alveolar remodeling that is common in severe asthma and in some instances of COPD (9). This suggests that adenosine may regulate aspects of collagen deposition in these diseases. Consistent with this, relatively high levels of adenosine are found in the lungs of asthmatic and COPD patients in association with severe inflammation (22, 23). In addition, adenosine levels are elevated in a mouse model of chronic pulmonary inflammation and remodeling (51). Mice overexpressing the pleuripotent cytokine IL-13 in the lungs develop features of chronic lung disease, including excessive collagen deposition around the airways (32). Interestingly, treatment of IL-13-overexpressing mice with ADA enzyme therapy was able to lower the levels of endogenous adenosine in the lungs and attenuate the degree of collagen deposition seen (51). These findings demonstrate that, as in the current study, elevations in lung adenosine levels can activate pathways that contribute to collagen deposition. Collectively, these studies suggest that controlling adenosine levels with exogenous ADA treatments may provide a meaningful approach to halt the progression or reverse features of pulmonary fibrosis not only in IPF but also in severe asthma and COPD. More research is needed to understand the regulation of adenosine metabolism in these chronic lung disorders, as well as to uncover the specific mechanisms by which adenosine exerts it proinflammatory and profibrotic activities. Doing so will not only build confidence in the potential use of ADA enzyme therapy in these diseases but identify specific targets for modulating adenosine’s actions.

Several studies of acute injury in the lung and in other tissues have shown that adenosine has potent anti-inflammatory and tissue protective effects (64–67). In contrast, the current study suggests that adenosine has proinflammatory, profibrotic, and tissue destructive effects on chronic lung injury. It is likely that differential responses of adenosine will be governed by the extent and duration of adenosine production, the specific expression of ARs on various effector cells, and the differential regulation of downstream signal transduction pathways. Deciphering these parameters in the context of the whole animal will be essential and will be aided by the emerging availability of mice with genetic modifications in components of adenosine metabolism and signaling and the increasing availability of selective AR ligands. Continued efforts to understand the contribution of adenosine signaling in both acute and chronic aspects of tissue inflammation and injury will be essential for the development of adenosine-based therapeutic approaches for the management of numerous disorders.

Acknowledgments
We thank Greg L. Shipley for his assistance with the quantitative RT-PCR experiments and Eva Morschl for her critical review of the manuscript.

Disclosures
The authors have no financial conflict of interest.

References


