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Ecto-5′-Nucleotidase (CD73)-Mediated Adenosine Production Is Tissue Protective in a Model of Bleomycin-Induced Lung Injury[1]

Jonathan B. Volmer,*‡ Linda F. Thompson,† and Michael R. Blackburn[2]*†‡

Adenosine signaling has diverse actions on inflammation and tissue injury. Levels of adenosine are rapidly elevated in response to tissue injury; however, the mechanisms responsible for adenosine production in response to injury are not well understood. In this study, we found that adenosine levels are elevated in the lungs of mice injured by the drug bleomycin. In addition, increased activity of ecto-5′-nucleotidase (CD73) was found in the lungs in conjunction with adenosine elevations. To determine the contribution of CD73 to the generation of adenosine in the lung, *CD73*$^{-/-}$ mice were subjected to bleomycin challenges. Results demonstrated that *CD73*$^{-/-}$ mice challenged with bleomycin no longer accumulated adenosine in their lungs, suggesting that the primary means of adenosine production following bleomycin injury resulted from the release and subsequent dephosphorylation of adenine nucleotides. *CD73*$^{-/-}$ mice challenged with bleomycin exhibited enhanced pulmonary inflammation and fibrosis as well as exaggerated expression of proinflammatory and profibrotic mediators in the lung. Intranasal instillations of exogenous nucleotidase restored the ability of lungs of *CD73*$^{-/-}$ mice to accumulate adenosine following bleomycin challenge. Furthermore, these treatments were associated with a decrease in pulmonary inflammation and fibrosis. *CD73*$^{+/+}$ animals challenged with bleomycin and supplemented with exogenous nucleotidase also exhibited reduced inflammation. Together, these findings suggest that CD73-dependent adenosine production contributes to anti-inflammatory pathways in bleomycin-induced lung injury. ***The Journal of Immunology,*** **2006, 176: 4449–4458.**

Tissue damage leads to the release of a wide variety of mediators that can influence local inflammatory responses. One such mediator is the signaling nucleoside adenosine, which is elevated at sites of tissue damage resulting from inflammation (1) or hypoxia (2, 3). By engaging specific adenosine receptors (AR),[3] this nucleoside exhibits both tissue-protective and destructive effects (4–6). For example, the production of adenosine in tissues can prevent damage resulting from ischemic reperfusion injury (7, 8), and adenosine signaling serves anti-inflammatory functions in response to certain endotoxic challenges (9). In contrast, adenosine can promote mast cell degranulation (10), induce bronchoconstriction (11), and exacerbate tissue injury in the lung (6) and brain (12). The differential actions of adenosine are likely mediated by the levels of ligand produced, and the specific pattern of AR expression on target cells. Substantial information is available regarding the regulation of ARs in inflammatory environments (5); however, relatively little is known about the mechanisms by which adenosine is produced following injury.

Elevations in extracellular adenosine can result from either an increase in intracellular adenosine followed by release into the extracellular space, or by the release of adenine nucleotides followed by their extracellular catabolism into adenosine (13). Intracellularly, adenosine can be generated from the hydrolysis of *S*-adenosylhomocysteine (14), or by the dephosphorylation of AMP by cytosolic nucleotidases (15). Extracellularly, adenosine can be generated following the release and dephosphorylation of adenine nucleotides (13). ATP and AMP are released from activated granulocytes (16, 17), and ADP is released from platelets upon degranulation (18). Bronchial epithelium can release ATP under basal conditions (19) and upon perturbation of the plasma membrane (20–22). Extracellular ATP and ADP can be converted into AMP by extracellular apyrases such as CD39 and alkaline phosphatase (23–25), and AMP can then be converted into adenosine by ecto-5′-nucleotidase (CD73) or alkaline phosphatase (24, 25). Examining the contribution of these various pathways to the generation of extracellular adenosine following injury could provide insight into novel approaches to control the production of this potent regulator of inflammation and tissue damage.

Emerging evidence suggests that concerted mechanisms likely exist for increased extracellular adenosine formation. CD73 forms adenosine from the dephosphorylation of extracellular AMP (26). CD73 is up-regulated following hypoxia as part of a response to increase local extracellular adenosine levels (27–29). This mechanism has been validated by subjecting CD73-deficient mice to hypoxic conditions and demonstrating that endothelial barrier function is compromised (30). Other studies have shown that CD73 contributes to adenosine-mediated effects on coronary blood vessels (31) and in the kidney (32). Thus, the dephosphorylation of

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[3] Abbreviations used in this paper: AR, adenosine receptor; APCP, $\alpha\beta$-methylene ADP; AMPase, AMP hydrolytic activity; OPN, osteopontin; PAI-1, plasminogen activator inhibitor-1; TIMP-1, tissue inhibitor of metalloproteinase-1; BAL, bronchial alveolar lavage; PEG, polyethylene glycol; P-NT, polyethylene glycol-modified nucleotidase.

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extracellular AMP by CD73 may represent a major route of adenosine formation in tissue injury.

Adenosine levels are elevated in the lungs of asthmatics (33, 34), and in various animal models of chronic lung disease (35–37). However, the mechanisms governing adenosine formation in the injured lung are not known. The major focus of this study was to examine the contribution of CD73 to adenosine formation following injury in the lung. This was accomplished by subjecting CD73-deficient mice to bleomycin-induced lung injury. Results demonstrated that exposure of mice to bleomycin results in elevated adenosine levels in conjunction with elevations in CD73 enzymatic activity. Exposure of CD73-deficient mice to bleomycin demonstrated that this enzyme is responsible for nearly all the adenosine formed in response to bleomycin-induced lung injury. Moreover, the lack of adenosine formation in bleomycin-treated CD73-deficient mice was associated with enhanced inflammation and fibrosis, suggesting that adenosine is protective in this model.

Materials and Methods

Mice

Genotypes of *CD73* mice (30) backcrossed onto C57BL/6J to the fifth generation were tracked with the following primers: wild-type allele, sense primer 5′-CTCTGCATTGCAGCCTGAAG-3′ and antisense primer 5′-CGATGTCCACACCTCGAACT-3′; knockout allele, sense 5′-CCAGCT CATTCCTCCCACTCATG-3′ and antisense 5′-CCATTTGAATAC TAGCTTCCAGG-3′. Six to 8-wk-old female C57/BL6J mice were purchased from Harlan. All mice were maintained and housed in accordance with the Animal Care Committee at the University of Texas Health Science Center at Houston and National Institutes of Health guidelines. 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 serologies on cage littermates were negative for 12 of the most common murine viruses.

Bleomycin exposures

Animals homozygous for the CD73 null allele were designated $CD73^{-/-}$. Animals homozygous for the CD73 wild-type allele were designated $CD73^{+/+}$. Six to 8-wk-old male and female littermate $CD73^{+/+}$ and $CD73^{-/-}$ mice from heterozygous mating pairs were used. Mice anesthetized with avertin were tracheostomized, and 3.5 U/kg bleomycin (Blenoxane; Bristol-Myers Squibb) diluted in 60 μl of normal saline or 60 μl of normal saline alone was instilled intratracheally. Endpoints were measured 14 days postchallenge.

Quantification of lung adenosine levels

Mice were anesthetized, and the lungs were rapidly removed and frozen in liquid nitrogen. Adenine nucleosides were extracted from frozen lungs using 0.4 N perchloric acid as described previously (38), and adenosine was separated and quantified using reversed phase HPLC.

Measurement of CD73 enzyme activity and Western blotting

Mice were anesthetized, and lungs were removed and frozen in liquid nitrogen. Membrane fraction proteins were extracted and used to assay CD73 protein levels by Western blot. CD73-specific activity was also measured in membrane fractions using the following procedure: 1 μg of total protein was preincubated at room temperature with 200 nM deoxycoformycin in 0.1 M HEPES (pH 7.4), with 50 μM MgCl, with or without $\alpha\beta$-methylene ADP (APCP; Sigma-Aldrich). Next, samples were incubated at 37°C for 30 min in the presence of 100 μM AMP. AMP hydrolytic activity (AMPase) was measured by determining adenosine concentrations with reversed phase HPLC (38). Western analysis was conducted using Ab generated against the peptide sequence NDVHSRLEQTSDDSTK, near the N terminus (Bethyl Laboratories). One hundred micrograms of membrane fraction protein per lane were run on a 7.5% polyacrylamide gel and transferred to a PVDF membrane for analysis via chemiluminescence.

RNA Extraction

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. Analysis of α1-procollagen, osteopontin (OPN), IL-1β, TGF-β1, TNF-α, plasminogen activator inhibitor-1 (PAI-1), and tissue inhibitor of metalloproteinase-1 (TIMP-1) was performed using quantitative RT-PCR as described previously (37, 39). CD73 transcript was measured by quantitative RT-PCR using primers specific for the CD73 mRNA (sense, 5′- TTGGCAAATAC CTGGGCTAC-3′; antisense 5′- AGGTTTCCCATGTTGCATTC-3′).

Histological analysis

Mice were anesthetized, and the lungs were perfused with 5–10 ml of PBS and then pressure infused with 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.

Bronchial alveolar lavage (BAL) and cellular differentials

Mice were anesthetized and tracheally intubated with a blunted 21-gauge needle. Lungs were lavaged with 1–2 ml of PBS, and the recovered BAL fluid was processed for the determination of cellular differentials. Briefly, total cell counts were performed on initial lavaged aliquots, and cellular differentials (300 cells/sample) were conducted on cells cytospun onto slides and stained with Diff-Quick (Dade Behring).

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 25°C. Homogenate was spun at 4000 rpm, and supernatant was assayed for pepsin soluble collagen according to the manufacturer's instructions.

Ashcroft scoring

Assessment of pulmonary fibrosis was performed on Masson's Trichrome-stained lung sections using a minor modification of the method outlined by Ashcroft et al. (40). For our purposes we analyzed 25 fields at ×40 per slide using a two-person randomized blind study. At least five mice were used for each group.

Polyethylene glycol (PEG)-conjugated nucleotidase

Crude venom from *Crotalus atrox* (Sigma-Aldrich) was resuspended in normal saline, and nucleotidase activity was extracted by affinity purification over AMP-Sepharose (Sigma-Aldrich). Weakly bound activity was extracted with 0.1% Triton in saline, and tightly bound activity was extracted with 10 mM AMP. Fractions were pooled and diluted in sterile PBS (pH 9.0), and 40 mg/ml mPEG-SPA (molecular mass 20,000 kDa; Nektar Therapeutics) was added and mixed at room temperature for 4 h. Pegylation efficiency was assessed by zymogram analysis as described previously (41), using AMP as a substrate. Endotoxin was removed using AffinityPak Endotoxin Columns (Pierce). Final product was filter sterilized by passage through a 0.2-μm filter. PEG-modified nucleotidase (P-NT) activity was determined by assaying AMPase by HPLC.

Nucleotidase replacement

Three hours after bleomycin challenge, $CD73^{+/+}$ or $CD73^{-/-}$ mice were anesthetized with isoflurane and instilled intranasally with 0.5 U of P-NT or PEG alone in 30 μl of normal saline (1 U is defined as activity for the generation of 1 μm adenosine from AMP per min at 37°C). Treatment was repeated on days 4, 8, and 12 of the challenge. Mice were sacrificed and endpoints measured on day 14 after bleomycin challenge.

Results

Adenosine accumulates in the lungs following bleomycin challenge in conjunction with increases in CD73 activity

To determine whether adenosine generation is altered during the pathogenesis of bleomycin-induced lung injury, adenosine levels and CD73 enzymatic activity were measured following bleomycin challenge. Mice challenged with bleomycin exhibited a 3-fold increase in lung adenosine levels compared with saline-treated controls (Fig. 1*A*). This elevation was coupled with a similar increase in AMPase (Fig. 1*B*) in membrane fractions isolated from the lungs of bleomycin-challenged mice. To distinguish CD73 from other AMPase, a specific inhibitor (APCP) was used. Both CD73 and non-CD73 AMPase were elevated upon bleomycin challenge,

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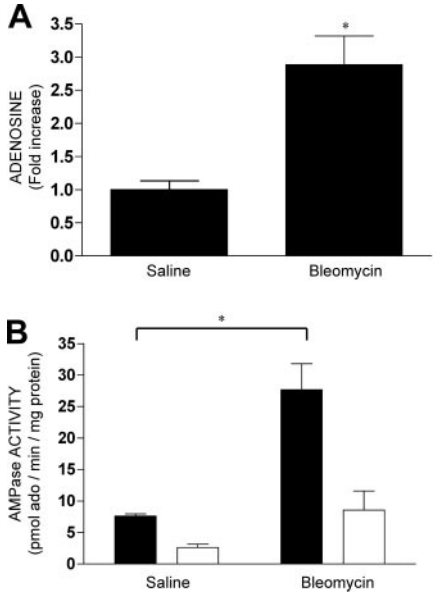

FIGURE 1. Bleomycin challenge elevates adenosine and CD73 activity levels. *A*, Adenosine levels in whole lung extracts 14 days after challenge with bleomycin or saline. Data are presented as mean fold increases ± SEM ($n = 8$; *, $p < 0.05$). *B*, AMPase in lung membrane fractions with (□) or without (■) APCP. Data are presented as mean picomoles of AMP converted to adenosine per minute per milligram of protein ± SEM ($n = 8$; *, $p < 0.05$).

with ~70% of the AMPase attributed to CD73. These findings demonstrate that adenosine levels are elevated following bleomycin challenge in association with increases in CD73-specific activity.

CD73$^{-/-}$ mice do not exhibit compensational activity from other AMPases

CD73-deficient mice were used to determine the contribution of CD73 to the production of adenosine in the lung (30). CD73-deficient (*CD73$^{-/-}$*), heterozygous (*CD73$^{+/-}$*), and wild-type (*CD73$^{+/+}$*) mice were examined for the presence of CD73 transcript and protein, as well as CD73 and non-CD73 AMPase, distinguished by sensitivity to the CD73-specific inhibitor, APCP. Quantitative RT-PCR on whole lung RNA extracts revealed no CD73 transcripts in *CD73$^{-/-}$* mice, and a reduction in CD73 transcripts in *CD73$^{+/-}$* mice (Fig. 2*A*). Similarly, Western blots of lung membrane fractions did not detect CD73 protein in *CD73$^{-/-}$* mice (Fig. 2*C*). Total AMPase in lung membrane fractions was reduced to non-APCP inhibitable levels, whereas non-CD73 activity was unaltered in the lungs of *CD73$^{-/-}$* mice (Fig. 2*B*). These findings demonstrate the absence of CD73 activity in the lungs of *CD73$^{-/-}$* mice, and show that there is no compensatory increase in activity of other nucleotidases.

CD73 is responsible for adenosine accumulation in the lungs following bleomycin injury

To determine whether CD73 activity is required for the accumulation of adenosine following bleomycin challenge, *CD73$^{-/-}$* mice were challenged with bleomycin, and nucleosides were quantified (Fig. 3). Whereas *CD73$^{+/+}$* mice exhibited a 3-fold increase in lung adenosine levels, no significant increase in adenosine was seen in the lungs of *CD73$^{-/-}$* mice. These data demonstrate that CD73 is responsible for adenosine accumulation that occurs following bleomycin-induced lung injury.

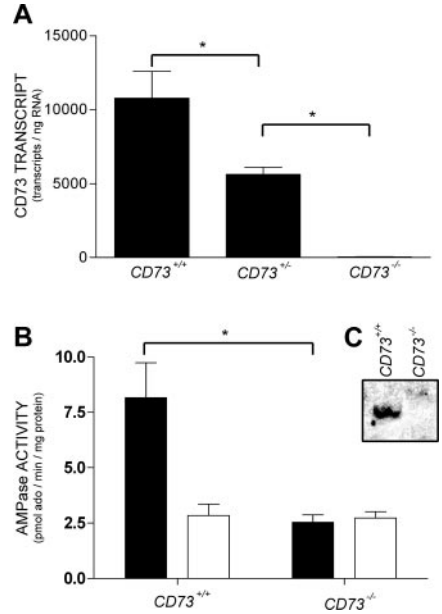

FIGURE 2. *CD73$^{-/-}$* mice lack CD73 transcript, protein, and activity in lungs. *A*, CD73 transcript levels in whole lung RNA extracts from *CD73$^{+/+}$*, *CD73$^{+/-}$*, and *CD73$^{-/-}$* mice. Data are presented as mean transcripts per nanogram of RNA ± SEM (*, $p < 0.05$; $n = 5$). *B*, AMPase in lung membrane fractions from *CD73$^{+/+}$* or *CD73$^{-/-}$* mice with (□) or without (■) APCP. Data are presented as mean picomoles of AMP converted to adenosine per minute per milligram of protein ± SEM (*, $p < 0.05$; $n = 5$). *C*, CD73 protein visualized by Western blot analysis of lung lysates from CD73$^{+/+}$ or CD73$^{-/-}$ mice.

Inflammatory endpoints are exacerbated in CD73$^{-/-}$ mice challenged with bleomycin

To determine the role of adenosine production on inflammation following bleomycin challenge, CD73$^{+/+}$ and CD73$^{-/-}$ mice were challenged with bleomycin and examined for inflammatory endpoints (Fig. 4). Histological examination revealed little to no interstitial inflammation in *CD73$^{+/+}$* or *CD73$^{-/-}$* mice challenged with saline (Fig. 4, *A* and *B*). Interstitial inflammation was evident in *CD73$^{+/+}$* mice challenged with bleomycin, but the inflammation was sparse and diffuse (Fig. 4*C*). In contrast, *CD73$^{-/-}$* mice challenged with bleomycin exhibited increased numbers of inflammatory foci in the distal airways (Fig. 4*D*). Although there were no histologically apparent differences between *CD73$^{+/+}$* and *CD73$^{-/-}$* mice following saline challenge, analysis of BAL cellularity revealed a slight but significant elevation in baseline inflammation in the lungs of *CD73$^{-/-}$* mice (Fig. 4, *E* and *F*). Following bleomycin challenge, there were elevations in cells

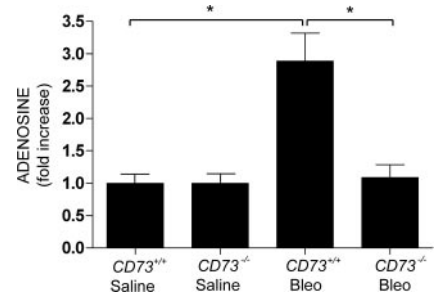

FIGURE 3. CD73 is the source of adenosine generated upon bleomycin challenge. Whole lung adenosine levels were determined in *CD73$^{+/+}$* and *CD73$^{-/-}$* mice 14 days after challenge with bleomycin (Bleo) or saline. Data are presented as mean fold increases ± SEM (*, $p < 0.05$; $n = 8$).

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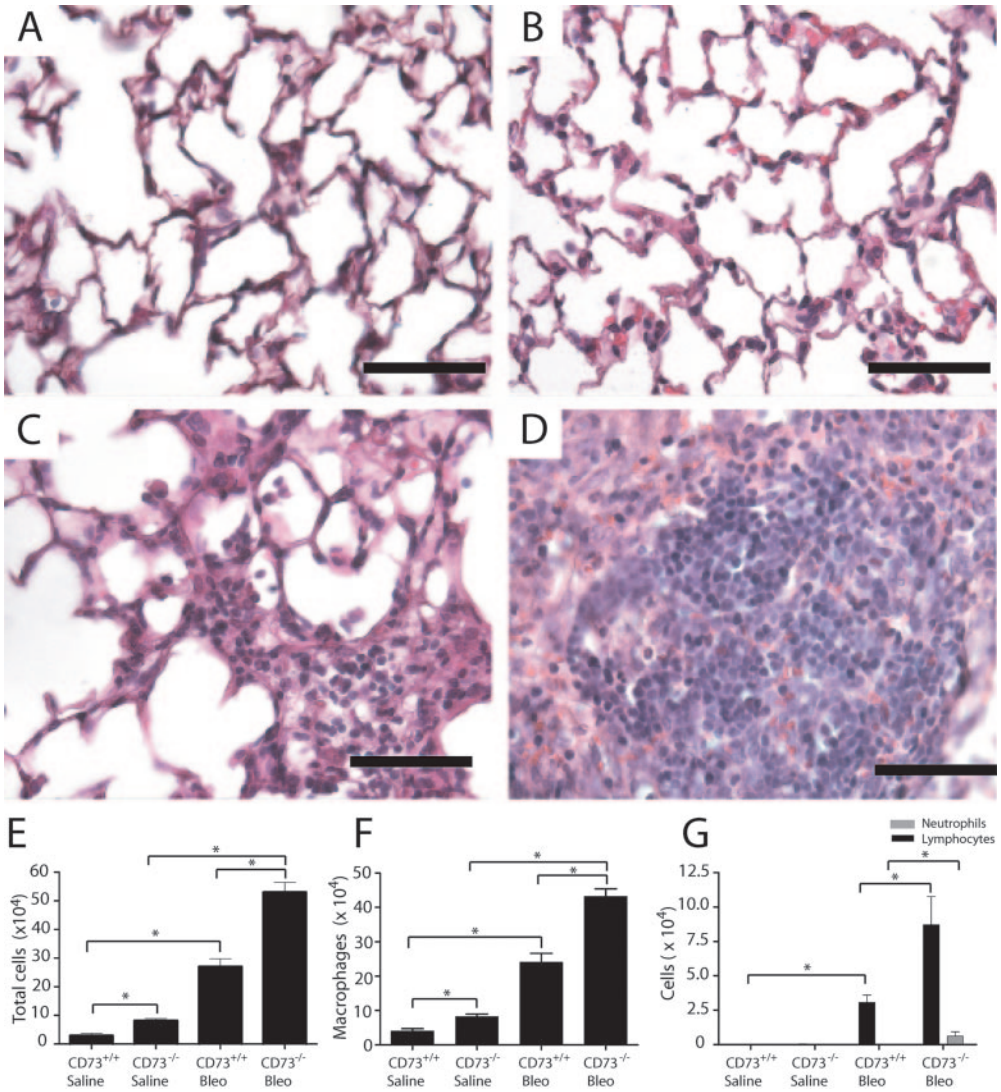

FIGURE 4. *CD73*$^{-/-}$ mice challenged with bleomycin exhibit enhanced inflammation. *CD73*$^{+/+}$ (*A* and *C*) or *CD73*$^{-/-}$ (*B* and *D*) mice 14 days after challenge with bleomycin (*C* and *D*) or saline (*A* and *B*). H&E staining; scale bars, 100 μm. Findings are representative of seven mice from each group. Total cells (*E*) or macrophages (*F*) recovered by BAL fluid ± SEM (*, $p < 0.05$; $n = 5$). *G*, Neutrophils (▨) and lymphocytes (■) from BAL fluid. Data are presented as mean total cells ± SEM (*, $p < 0.05$; $n = 7$).

recovered in the BAL of *CD73*$^{+/+}$ mice, and these increases were enhanced in the BAL fluid of *CD73*$^{-/-}$ mice (Fig. 4, *E–G*). *CD73*$^{-/-}$ mice challenged with bleomycin exhibited about a 2.5-fold greater level of total cells recovered in the BAL, as well as significant increases in macrophages, lymphocytes, and neutrophils (Fig. 4, *E–G*). These data demonstrate that loss of CD73 results in enhanced pulmonary inflammation following bleomycin challenge.

Collagen production and deposition is exacerbated in CD73$^{-/-}$ *mice challenged with bleomycin*

Collagen overproduction and deposition are prominent features of bleomycin-induced pulmonary fibrosis (42). Masson's Trichrome staining revealed increased collagen deposition (blue staining) in the lungs of *CD73*$^{+/+}$ mice following bleomycin challenge (Fig. 5, *A* and *C*). The degree of collagen deposition was enhanced in the lungs of *CD73*$^{-/-}$ mice challenged with bleomycin (Fig. 5*D*). Consistent with these observations, analysis of whole lung RNA revealed an increase in α-1-procollagen transcript in bleomycin-treated *CD73*$^{-/-}$ mice (Fig. 5*E*). Furthermore, total collagen content was enhanced in the lungs of *CD73*$^{-/-}$ mice challenged with bleomycin (Fig. 5*F*). These findings demonstrate that loss of CD73 results in enhanced collagen production and deposition in the lung following exposure to bleomycin.

To asses the overall magnitude and distribution of fibrosis in the lungs of *CD73*$^{-/-}$ mice, Ashcroft scoring was conducted. This indicator of general inflammation and fibrosis provided another means of demonstrating that bleomycin-challenged *CD73*$^{-/-}$ mice have enhanced pulmonary fibrosis (Fig. 6*A*). Lastly, as a result of the combined effects of exacerbated inflammation and fibrosis, *CD73*$^{-/-}$ mice exhibited a significant reduction in survival following bleomycin challenge (Fig. 6*B*). These data demonstrate that loss of CD73 is associated with an exacerbation of bleomycin-induced lung injury.

Enhanced expression of proinflammatory and profibrotic mediators in CD73$^{-/-}$ *mice challenged with bleomycin*

Enhanced pulmonary inflammation and fibrosis in the lungs of *CD73*$^{-/-}$ mice treated with bleomycin prompted us to examine levels of inflammatory and fibrotic mediators (Fig. 7). Transcript levels for the proinflammatory mediators IL-1β (Fig. 7*A*) and TNF-α (Fig. 7*B*) were found to be elevated in RNA extracts from

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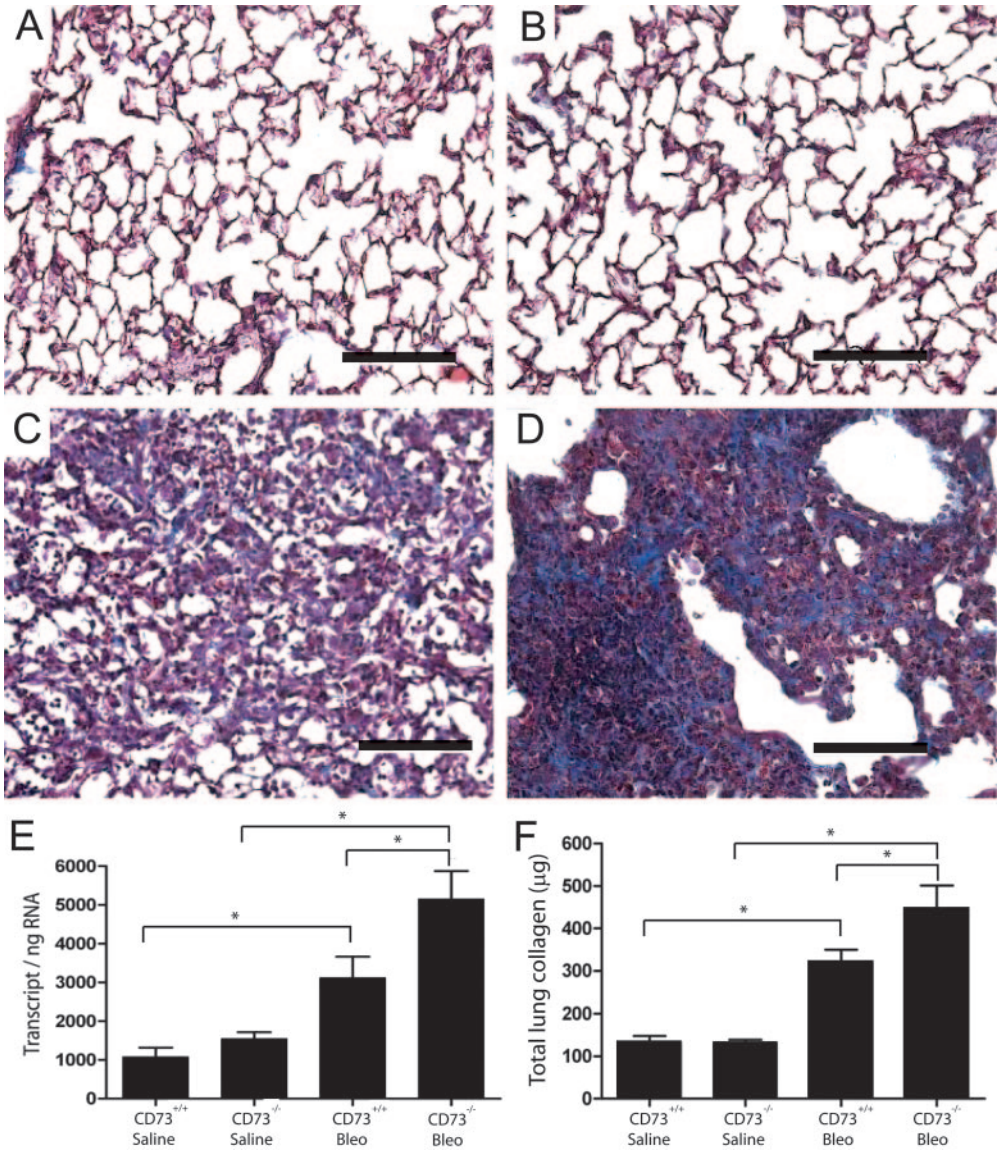

FIGURE 5. *CD73*$^{-/-}$ mice challenged with bleomycin exhibit enhanced collagen production. *CD73*$^{+/+}$ (*A* and *C*) or *CD73*$^{-/-}$ (*B* and *D*) mice 14 days after challenge with bleomycin (*C* and *D*) or saline (*A* and *B*). Masson's Trichrome staining; scale bars, 100 μm. Findings are representative of seven mice from each group. Collagen levels were quantified by (*E*) α-1-procollagen transcripts in whole lung (mean transcripts per nanogram of RNA $\pm$ SEM; $*$, $p < 0.05$; $n = 5$) and (*F*) collagen protein levels as determined by the Sircol assay on whole lung homogenates (presented as mean micrograms $\pm$ SEM; $*$, $p < 0.05$; $n = 6$).

CD73$^{+/+}$ mice treated with bleomycin. Furthermore, the levels of these mediators were significantly higher in RNA extracts from the lungs of *CD73*$^{-/-}$ mice treated with bleomycin (Fig. 7, *A* and *B*). Similarly, levels of profibrotic mediators such as TGF-β1 (Fig. 7*C*), OPN (Fig. 7*D*), PAI-1 (Fig. 7*E*), and TIMP-1 (Fig. 7*F*) were enhanced in the lungs of *CD73*$^{-/-}$ mice treated with bleomycin. These data demonstrate that loss of CD73 is associated with enhanced expression of proinflammatory and profibrotic mediators in the lung following bleomycin exposure.

Restoration of AMPase activity to the lungs of CD73$^{-/-}$ *mice reduces inflammation and fibrosis*

To verify that the absence of CD73 AMPase activity is responsible for the observed effects in *CD73*$^{-/-}$ mice, exogenous nucleotidase conjugated to PEG (P-NT) was introduced to the lungs of *CD73*$^{-/-}$ and *CD73*$^{+/+}$ mice following bleomycin challenge. Intranasal administration of P-NT to the lungs of *CD73*$^{-/-}$ mice reduced total cell infiltrates, macrophages, and lymphocytes to levels comparable to those seen in *CD73*$^{+/+}$ mice (Fig. 8). Interestingly, introduction of P-NT to the lungs of *CD73*$^{+/+}$ mice reduced total cellular infiltrates after bleomycin challenge (Figs. 8 and 9). Similarly, treatment with P-NT resulted in a decrease in collagen deposition and overall fibrosis in the lungs of *CD73*$^{-/-}$ mice (Fig. 9). These data demonstrate that treatment with exogenous nucleotidase can reverse the effects of CD73 deficiency on bleomycin-induced lung injury.

Treatment with exogenous nucleotidase elevates lung adenosine levels following bleomycin challenge

Adenosine levels were measured in mice administered P-NT to ensure that exogenous nucleotidase treatment was affecting adenosine levels in the lungs of bleomycin-treated mice. Adenosine levels were found to be elevated in both *CD73*$^{+/+}$ and *CD73*$^{-/-}$ mice following P-NT treatment and bleomycin challenge, but not after saline challenge (Fig. 10). These data demonstrate the ability of P-NT to elevate adenosine levels in *CD73*$^{-/-}$ mice, suggesting

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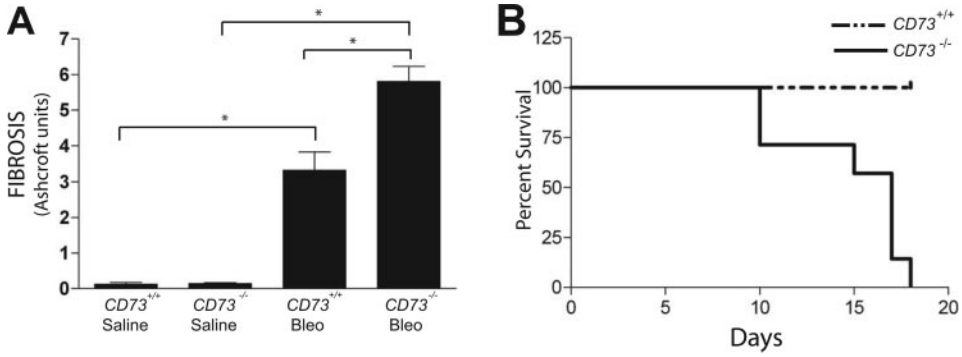

FIGURE 6. Fibrosis is more severe, and survival is reduced in $CD73^{-/-}$ mice challenged with bleomycin. Severity of the response to bleomycin challenge was assessed by Ashcroft scoring on day 14 after bleomycin challenge (mean Ashcroft score ± SEM (*A*); ∗, $p < 0.05$; $n = 8$) and survival (*B*; $n = 7$).

that improvements in bleomycin-induced injury following P-NT treatments are due to elevations in adenosine levels. Furthermore, the observation that introduction of additional AMPase activity can increase adenosine levels in $CD73^{+/+}$ mice suggests that the conversion of AMP into adenosine is the rate-limiting step in the generation of adenosine in this model.

Discussion

Adenosine is a ubiquitous nucleoside that is typically found at low concentrations in the extracellular space; however, levels can rise substantially in response to tissue injury or metabolic stress (5, 43). Elevations in extracellular adenosine dictate various cellular responses by engaging ARs. Thus, examining the mechanisms by which extracellular adenosine levels are regulated is important for understanding how this nucleoside regulates tissue protection, injury, and repair. Adenosine can be produced both intracellularly and extracellularly by a wide array of enzymes and overlapping pathways (13). Intracellularly, adenosine can be formed by the dephosphorylation of intracellular adenine nucleotide pools by the cytosolic nucleotidases (15), or by the hydrolysis of *S*-adenosylhomocysteine (14). Once formed, adenosine can diffuse into the extracellular space via equilibrative nucleoside transporters (44). Metabolic stress can increase the rate at which adenosine is formed by these pathways, potentially causing an accumulation of adenosine extracellularly. Alternatively, adenosine can be formed in the extracellular space by the dephosphorylation of adenine nucleotides released from cells (13). It is unclear which pathway and which enzymes are responsible for extracellular adenosine accumulations following tissue injury. The

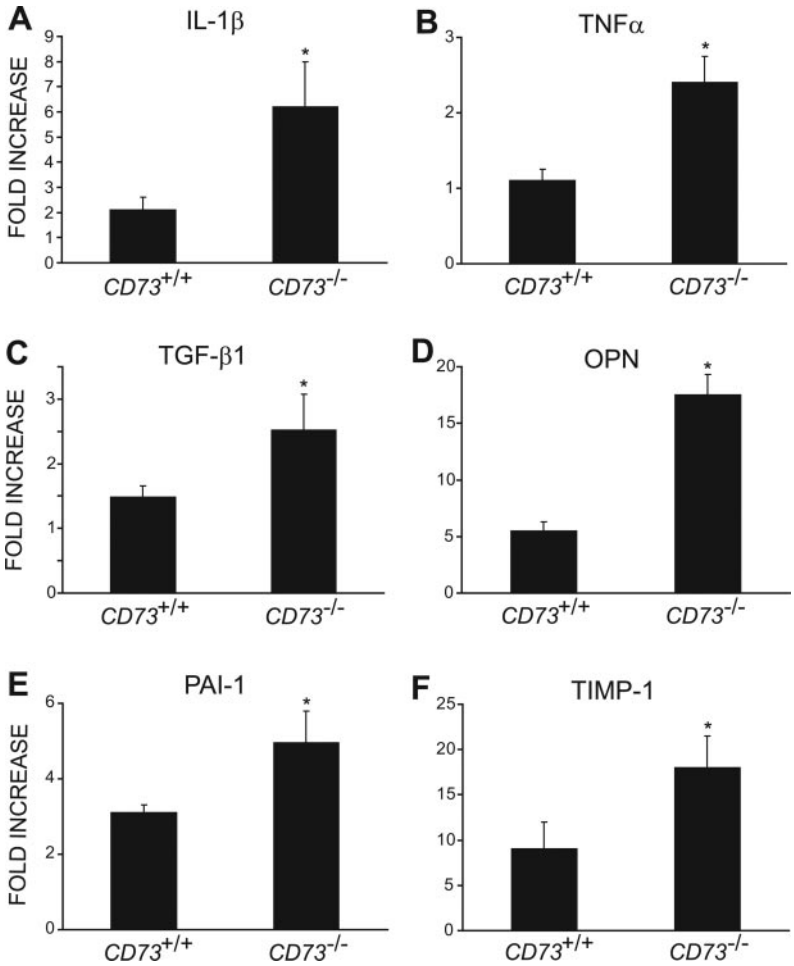

FIGURE 7. Expression of inflammatory and fibrotic mediators in the lung. RNA was isolated from the lungs of $CD73^{-/-}$ or $CD73^{+/+}$ mice 14 days after exposure to saline or bleomycin and subjected to quantitative RT-PCR for various inflammatory and fibrotic mediators. *A*, IL-1β; *B*, TNF-α; *C*, TGF-β1; *D*, OPN; *E*, PAI-1; *F*, TIMP-1. Data are presented as mean fold increases in transcript levels ± SEM in RNA extracts from bleomycin-treated mice compared with saline-treated mice (∗, $p < 0.05$; $n = 6–8$).

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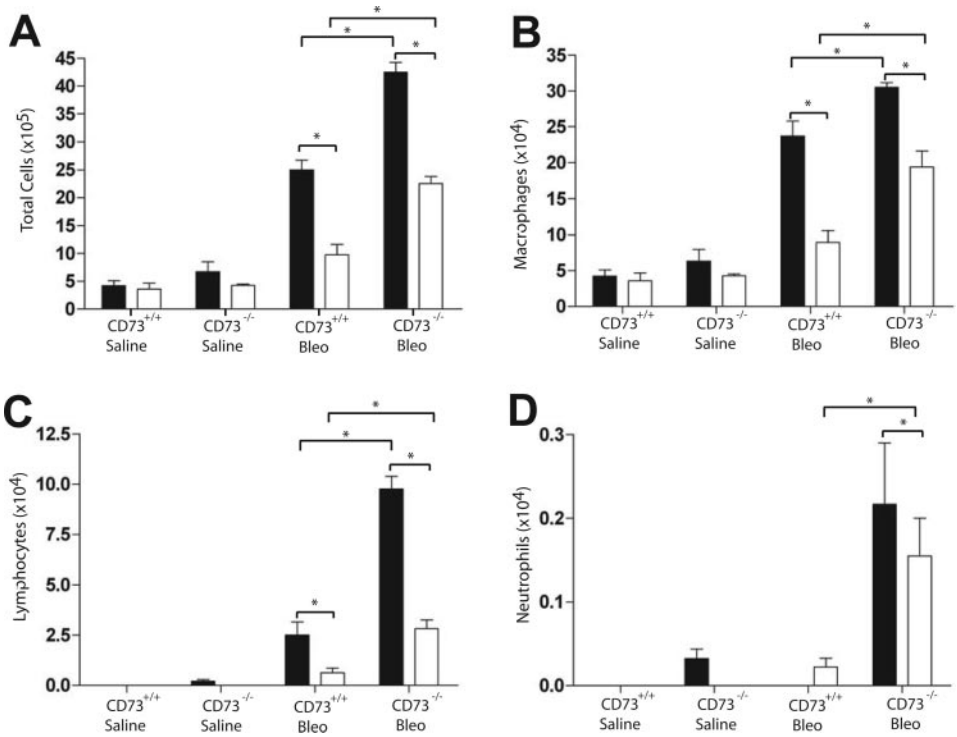

FIGURE 8. Restoration of AMPase improves inflammatory status. Total cells (*A*), macrophages (*B*), lymphocytes (*C*), and neutrophils (*D*) recovered from the BAL fluid of *CD73*$^{+/+}$ or *CD73*$^{-/-}$ mice, challenged with bleomycin or saline and treated with P-NT (□) or vehicle (■). Data are presented as mean total cells ± SEM (*, $p < 0.05$; $n = 5$).

current study demonstrates that in bleomycin-induced lung injury, adenosine elevations arise from the breakdown of extracellular nucleotides, and that CD73 is critical in the formation of adenosine from these released nucleotides.

A major observation from this study was that the level of CD73 enzymatic activity is up-regulated in the lungs following bleomycin injury in conjunction with elevations in adenosine. This finding, together with the demonstration that deletion of CD73 results in loss of adenosine accumulation, suggests that CD73 up-regulation is an orchestrated and crucial response to regulating extracellular adenosine levels. The regulation of CD73 has been linked to factors that are consistent with this hypothesis. Hypoxia can lead to increased transcription and activity of CD73 (27, 28) in part through mechanisms that involve the transcription factor hypoxia-inducible factor-1α (27). Hypoxia is common in situations of tissue inflammation and injury, and is associated with adenosine elevations (3, 45). Furthermore, CD73 can be up-regulated by adenosine itself through transcriptional regulation via cAMP response element elements in the CD73 promoter (27). Such feed forward regulation of adenosine production emphasizes the necessity of orchestrated generation of this signaling nucleoside. The mechanism by which CD73 is up-regulated in response to bleomycin injury is not known, but the findings in this study provide important in vivo evidence for purinergic remodeling responses in the damaged lung that favor the production of adenosine. In addition, excessive apoptosis occurs in the airways following bleomycin challenge (46), which may contribute to adenosine generation; however, the observations that adenosine levels are elevated in both humans (33, 34) and mice (35–37) that exhibit features of asthma and chronic obstructive pulmonary disease, suggests that CD73-dependent adenosine generations may be a widespread feature of lung injury. Interestingly, nucleotidase activity is elevated in epithelial cells isolated from cystic fibrosis patients (47), and asthmatics have a unique sensitivity to inhaled AMP (11). Both of these findings suggest that regulated increases in CD73 may play a major role in adenosine-mediated effects in the inflamed lung.

Adenosine has been shown to have an array of differing and sometimes opposing inflammatory effects depending on the type of injury and the tissues involved (4–6, 48). We demonstrate in this study that reduced adenosine accumulation in the lungs of *CD73*$^{-/-}$ mice results in enhanced inflammation and fibrosis in response to bleomycin challenge. Inflammatory infiltrates and collagen deposition were increased in the lungs of *CD73*$^{-/-}$ mice treated with bleomycin, and survival was reduced. In addition, there was enhanced expression of key inflammatory and fibrotic mediators in the lungs of *CD73*$^{-/-}$ mice given bleomycin. The dependence of this response on the ability to produce adenosine was verified by the reintroduction of exogenous AMPase activity to *CD73*$^{-/-}$ mice. These findings suggest that adenosine elevations following bleomycin-induced injury are serving an anti-inflammatory and tissue-protective role. These observations are consistent with previous findings implicating an anti-inflammatory and tissue-protective role for CD73-mediated adenosine generation. Under hypoxic conditions, *CD73*$^{-/-}$ mice exhibit increased vascular leakage and neutrophil accumulation that is reduced by the administration of exogenous AMPase activity (30, 45). Increased vascular leakage was also observed when a specific inhibitor of CD73 (APCP) was acutely administered to normal mice under hypoxic conditions (27, 45). In addition, CD73-mediated adenosine production has been shown to play an important role in regulating inflammation in an air-pouch model of tissue injury (49). These findings, together with the results in the current study suggest that production of adenosine by CD73 is an important pathway for tissue protection.

Consistent with an anti-inflammatory/tissue-protective role for adenosine, we found that *CD73*$^{+/+}$ mice challenged with bleomycin and supplemented with exogenous AMPase activity exhibit reduced numbers of inflammatory cells in the BAL fluid. These findings suggest that elevating adenosine production through increasing nucleotidase activity is able to enhance the endogenous anti-inflammatory effects of adenosine. Another interesting observation was that the levels of BAL inflammatory cells in the lungs of unchallenged *CD73*$^{-/-}$ mice were elevated, suggesting that CD73-mediated adenosine production plays an important role in the homeostatic regulation of tissue inflammation by maintaining

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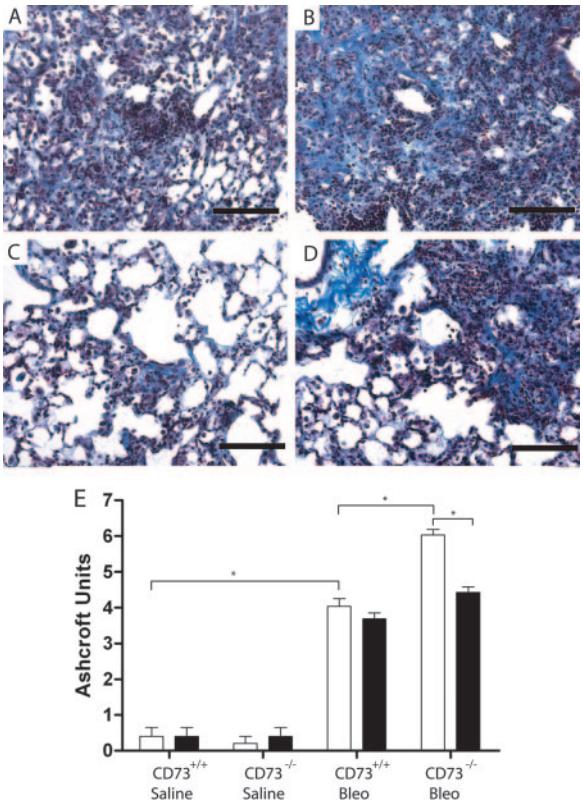

FIGURE 9. Restoration of AMPase reduces collagen production in the lungs of $CD73^{-/-}$ mice. Masson's Trichrome staining was performed on lung sections from $CD73^{+/+}$ (*A* and *C*) and $CD73^{-/-}$ (*B* and *D*) mice challenged with bleomycin and treated with P-NT (*C* and *D*) or vehicle (*A* and *B*); scale bars, 100 μm. Findings are representative of five mice from each group. To determine the severity of the fibrotic changes, Ashcroft scores were determined (*E*) in animals treated with P-NT (■) or vehicle (□). Data are presented as mean Ashcroft score ± SEM (*, $p < 0.05$; $n = 5$).

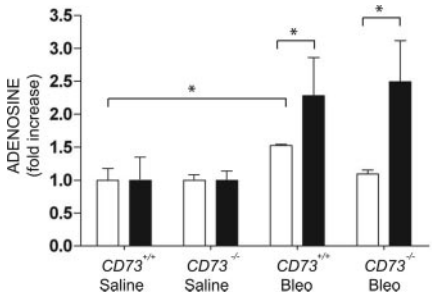

FIGURE 10. Adenosine levels are elevated following restoration of AMPase. Whole lung adenosine levels were measured in $CD73^{+/+}$ or $CD73^{-/-}$ mice challenged with bleomycin or saline and treated with P-NT (■) or vehicle (□). Data are presented as mean fold increases ± SEM (*, $p < 0.05$; $n = 6$).

endothelial integrity and preventing vessel leakage and leukocyte infiltration (30). These findings suggest that there are ample quantities of extracellular adenine nucleotides available for conversion into adenosine in the normal and injured lung, and that the availability of CD73 enzymatic activity on the cell surface is a limiting factor. Indeed, adenine nucleotides can be produced by multiple cell types including inflammatory cells (16–18) and airway epithelial cells (19–22), all of which can contribute to the tropic- and injury-induced production of adenosine by CD73. This raises the possibility of using exogenous nucleotidase-based therapies to regulate adenosine production to benefit certain inflammatory situations. However, additional studies are needed to categorize the effects of adenosine in various injury situations, because excessive or prolonged adenosine elevations may activate pathways that exacerbate inflammation and tissue injury (6).

Previous studies from our laboratory have shown that adenosine can serve as a profibrotic signal in the lung (37). Prolonged elevations in adenosine in the lungs of mice lacking adenosine deaminase are associated with inflammation and airway injury that includes extensive airway fibrosis (37). Furthermore, elevation in endogenous adenosine has profibrotic actions in the lungs of mice with Th2-induced lung disease (35). These studies suggest that elevations in adenosine are sufficient to access profibrotic pathways in the lungs. In contrast, the current study clearly demonstrates that elevations in adenosine are not necessary for the development of pulmonary fibrosis. Understanding this paradox likely lies in the appreciation of the diverse activities of the various ARs (43). The ARs are found on a variety of cells implicated in the regulation of lung inflammation and damage. The A_1AR is expressed on macrophages (39) and airway smooth muscle (50), and perhaps airway epithelial cells (39), the A_{2A}AR is present on endothelial cells, macrophages, neutrophils and lymphocytes (5), the A_3AR is present on macrophages (51), eosinophils (41, 52), mast cells (53), and mucus-producing epithelial cells (35, 41), and the A_{2B}AR seems to be ubiquitous, being found on all of the aforementioned cells (5, 54) as well as pulmonary fibroblasts and myofibroblasts (55). Whether engagement of these receptors is associated with anti-inflammatory/tissue-protective effects, or proinflammatory/tissue-destructive effects is dictated by the levels of ligand produced, the pattern of receptor expression on various cells, the effector systems coupled to these receptors, and the cytokine or growth factor environment (5, 54).

Anti-inflammatory and tissue-protective effects have been assigned to the A_1AR, A_{2A}AR, and A_3AR (4, 5, 48). The enhanced inflammation seen in association with the lack of adenosine production in the lungs of bleomycin-challenged $CD73^{-/-}$ mice might represent a loss of anti-inflammatory effects mediated by these ARs. The enhanced production of IL-1β and TNF-α in $CD73^{-/-}$ mice treated with bleomycin is consistent with such a hypothesis. Interestingly, TNF-α production has been shown to be decreased by engagement of the A_{2A}AR or A_3AR (51, 56, 57), suggesting that the absence of damaged-induced adenosine production and engagement of AR anti-inflammatory mechanisms contribute to the enhanced inflammation seen in the lungs of $CD73^{-/-}$ mice exposed to bleomycin. The subsequent augmentation of pulmonary fibrosis might result from increased generation of profibrotic mediators (TGF-β1, OPN, PAI-1, TIMP-1) as a result of the enhanced inflammatory response. In the case of adenosine-induced pulmonary fibrosis in other models, the involvement of proinflammatory and/or profibrotic actions of ARs might predominate over anti-inflammatory effects of adenosine. Recent studies have demonstrated that engagement of the A_{2B}AR can drive the transformation of pulmonary fibroblasts into myofibroblasts, suggesting that this receptor has profibrotic activities (55). Interestingly, the A_{2B}AR has a low affinity for adenosine (58), and might only be activated in situations with pronounced adenosine accumulations. Therefore, adenosine might serve anti-inflammatory tissue-protective roles at acute stages of injury, and as damage ensues and adenosine levels rise, profibrotic actions of the A_{2B}AR might contribute to the amplification and progression of pulmonary fibrosis. This theory would emphasize the need to maintain a balance in adenosine production and signaling to ensure proper tissue repair as opposed to propagation of fibrosis. Understanding the regulation and contribution of adenosine production and AR regulation over the course of tissue

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injury and repair will be paramount to deciphering how this balance is maintained.

In conclusion, we demonstrate that ecto-5′-nucleotidase (CD73) is needed for the generation of adenosine in the lungs of mice exhibiting bleomycin-induced inflammation and fibrosis. In addition, diminished adenosine generation in the lungs of bleomycin-challenged *CD73*$^{-/-}$ mice was associated with enhanced pulmonary inflammation and fibrosis, suggesting that adenosine has anti-inflammatory and tissue-protective actions in this model. Studies are in progress to assess the specific contribution of individual ARs in bleomycin-induced lung injury with the intent of deciphering the mechanisms associated with both anti-inflammatory/tissue-protective and proinflammatory/tissue-destructive contributions of adenosine to pulmonary fibrosis.

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Disclosures

M. R. Blackburn is a paid consultant with CV Therapeutics, which develops adenosine-based therapeutics.

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