Interleukin-33 Potentiates Bleomycin-Induced Lung Injury

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The mechanisms of interstitial lung disease (ILD) remain incompletely understood, although recent observations have suggested an important contribution by IL-33. Substantial elevations in IL-33 expression were found in the lungs of patients with idiopathic pulmonary fibrosis and scleroderma lung disease, as well as in the bleomycin injury mouse model. Most of the observed IL-33 expression was intracellular and intranuclear, suggesting involvement of the full-length (fl) protein, but not of the proteolytically processed mature IL-33 cytokine. The effects of flIL-33 on mouse lungs were assessed independently and in combination with bleomycin injury, using recombinant adenovirus-mediated gene delivery. Bleomycin-induced changes were not affected by gene deficiency of the IL-33 receptor T1/ST2. Combined flIL-33 expression and bleomycin injury exerted a synergistic effect on pulmonary lymphocyte and collagen accumulation, which could be explained by synergistic regulation of the cytokines transforming growth factor- β , IL-6, monocyte chemoattractant protein-1, macrophage inflammatory protein-1 α , and tumor necrosis factor- α . By contrast, no increase in the levels of the Th2 cytokines IL-4, IL-5, or IL-13 was evident. Moreover, flIL-33 was found to increase the expression of several heat shock proteins (HSPs) significantly, and in particular HSP70, which is known to be associated with ILD. Thus, flIL-33 is a synergistic proinflammatory and profibrotic regulator that acts by stimulating the expression of several non-Th2 cytokines, and activates the expression of HSP70.

Keywords: IL-33; interstitial lung disease; inflammation; fibrosis; lymphocytes

Interstitial lung disease (ILD), a combination of pulmonary inflammation and excessive scarring, or fibrosis, develops as a complication of various disease processes or idiopathically, as in the case of idiopathic pulmonary fibrosis (IPF). In patients with scleroderma or systemic sclerosis (SSc), scleroderma lung disease (SLD) is the leading cause of death (1, 2), whereas patients with IPF live an average of only 2–3 years after diagnosis (3). The mechanisms of ILD are not fully understood, and the available therapies are of limited effectiveness (4). Cytokines appear to play major roles in ILD through an intricate interplay between their proinflammatory and antiinflammatory as well as profibrotic and antifibrotic effects,

CLINICAL RELEVANCE

The findings of this study implicate full-length IL-33 as a regulator of pulmonary inflammation and fibrosis. In contrast to the effects of mature IL-33, which is known to drive asthma, allergy, and antiparasitic responses through the T1/ST2 receptor and Th2 mechanisms, full-length IL-33 remains predominantly intracellular and regulates the expression of genes that propel inflammation and fibrosis in the lungs.

which ultimately lead to excessive connective-tissue deposition (4–7). The bleomycin injury model is often used to investigate the cellular and molecular mechanisms of ILD, and despite its limitations, remains relevant because it recapitulates key features of the human disease (8). Among other advantages, the bleomycin model is useful in identifying endogenous profibrotic and antifibrotic molecules and testing antifibrotic drug candidates. For example, recent reports have implicated heat shock protein (HSP)–70 in pulmonary fibrosis in the bleomycin injury (9–11) and LPS injury (12) models. The bleomycin model can also be used to assess the ability of molecular mediators to enhance or attenuate bleomycin-induced inflammation and/or fibrosis in a “double-hit” setting (13).

IL-33, a member of the IL-1 family of cytokines, has recently emerged as a key regulator of inflammatory and immune processes (14–17). Proteolytic maturation of IL-33 and its binding to a cell-surface receptor composed of IL-1 receptor accessory protein and the T1/ST2 glycoprotein is necessary for the majority of its biological effects, and in particular, its potent pro-Th2 activity (18–20). In addition, recent data suggest that proteolytically uncleaved, full-length (fl)IL-33, which remains predominantly intracellular and, moreover, intranuclear, promotes inflammation in a T1/ST2-independent fashion through the regulation of gene expression (20). However, the full range of biological activities of flIL-33 is not well understood.

HSP70 has been suggested to contribute to ILD, but its exact roles need further clarification. Elevated HSP70 was noted in scleroderma fibroblasts (21, 22), and the levels of HSP70 in serum were associated with pulmonary fibrosis and inflammation in these patients (23, 24). HSP70 expression was strongly elevated in the surgical lung biopsies of patients with progressive IPF (25), and patients with IPF and anti-HSP70 autoantibodies were found to exhibit more near-term lung function deterioration and mortality (26). Conflicting initial data suggested either profibrotic (27, 28) or antifibrotic (9–12) regulation by HSP70.

We recently reported that flIL-33 expression is elevated in the lungs of patients with IPF, and that it induces pulmonary lymphocytic inflammation *in vivo* without engaging the T1/ST2 receptor and without activating the Th2 cytokine pattern (20). To assess the potential involvement of flIL-33 in ILD further, we compared IL-33 expression in the bleomycin injury model with human ILD, and investigated the effects of combined flIL-33

(Received in original form February 28, 2013 and in final form June 21, 2013)

This work was supported by Department of Veterans Affairs Merit Awards 5I01CX000101 (I.G.L.) and 5I01CX000107 (S.P.A.), by National Institutes of Health and National Institute of Arthritis and Musculoskeletal and Skin Diseases grant 1R03AR054946 (I.G.L.), by National Institutes of Health and National Heart, Lung, and Blood Institute grant 1R21HL106196 (S.P.A.), and by a Scleroderma Foundation Mark Flapan Award (I.G.L.).

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 49, Iss. 6, pp 999–1008, Dec 2013

Published 2013 by the American Thoracic Society

Originally Published in Press as DOI: 10.1165/rcmb.2013-0093OC on July 9, 2013

Internet address: www.atsjournals.org

gene delivery and bleomycin injury in mice. The results further implicate flIL-33 as an independent proinflammatory and profibrotic regulator that acts without eliciting the Th2 response, yet activates HSP70 expression and propels ILD by inducing the secretion of several cytokine mediators.

MATERIALS AND METHODS

Patients and Patient Samples

This study was reviewed and approved by the Institutional Review Board at the University of Maryland School of Medicine. Lung tissues from patients with IPF or SSc were obtained through video-assisted thoracoscopic biopsy or from pulmonary explants remaining after lung transplantation (29–37). Normal lung-tissue samples were obtained from lungs harvested initially for lung transplantation, but ultimately not used for transplantation purposes.

Experimental Animals and *In Vivo* and *In Vitro* Experiments

This study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Maryland. Experiments were performed in 10–12-week-old wild-type (WT) female C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME), T1/ST2-deficient ($^{-/-}$) female mice on a BALB/c background (a kind gift from Dr. Andrew

N. J. McKenzie, Medical Research Council Laboratory of Molecular Biology, Cambridge, UK) (20, 38), and, as a control for the T1/ST2-deficient strain, WT BALB/c mice. All animals were maintained in sterile microisolator cages with sterile rodent feed and acidified water. Daily maintenance of mice was performed at the Baltimore Veterans Affairs Medical Center Research Animal Facility, which is approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Adenoviral gene delivery was used to express fl mouse (flm) and mature mouse (mm) IL-33 in mouse lungs *in vivo*. Recombinant adenoviral vectors encoding flmIL-33 (AdV-flIL33), mmIL-33 (AdV-mmIL33), and a control vector not encoding a cytokine were constructed, manufactured, validated, and instilled intratracheally, as previously reported (13, 20, 35, 39–41). The resultant purified viruses presented a concentration of 1×10^{12} particles/ml and an infectious titer of 4×10^{10} plaque-forming units (PFU)/ml. Viruses were instilled at 5×10^8 PFU/mouse. In cell cultures, the same AdV constructs were used at 1×10^8 PFU/ml. Quantitative RT-PCR and ELISA of lung homogenates were used to confirm the expression of IL-33 mRNA and protein, respectively, as described elsewhere (20). Intratracheal instillations of 0.075 U of bleomycin (BLM; Sigma-Aldrich, St. Louis, MO) or PBS were performed as previously described (13, 20, 35, 39–41). Bronchoalveolar lavage (BAL) analyses, the preparation and analyses of pulmonary homogenates, histological and immunohistological analyses, multiplex analyses of cytokines, Western blotting, quantitative RT-PCR, ELISA, and human and

Figure 1. IL-33 expression in interstitial lung disease (ILD). (A) Immunohistochemical staining of lung tissues for IL-33, with positive staining appearing brown. Digital images were accumulated with $\times 20$ and $\times 60$ objectives, as indicated. Nonimmune control (Ctrl) IgG did not stain lung tissue (a section from a patient with idiopathic pulmonary fibrosis [IPF] is shown at upper left), and normal human lungs showed a moderate basal expression of IL-33 (upper right). By contrast, the lungs of patients with IPF (middle row) and systemic sclerosis (SSc; bottom row) showed an abundance of IL-33–positive cells, including infiltrating cells (arrows at $\times 20$ magnification) and stromal cells (arrowheads point to selected cells at $\times 60$ magnification). These experiments were repeated in three healthy control subjects, 11 patients with IPF, and four patients with SSc, with consistent results. (B) Quantitative RT-PCR results for IL-33 mRNA normalized to 18S ribosomal RNA show the fold-difference from the mean values for healthy control samples. Total RNA was purified from primary pulmonary fibroblast cultures derived from six healthy control subjects (light circles), six patients with IPF (dark gray circles), and four patients with scleroderma lung disease (medium gray circles). The difference in IL-33 mRNA expression between healthy control subjects (Ctrl) and patients with ILD was significant ($P = 0.031$, two-sided Mann-Whitney test). (C) Western blotting of primary human adult pulmonary fibroblast culture lysates from three separate donors (D1–D3), 48 hours after transfection with the plasmids encoding either human IL-33 or not encoding a cytokine (NULL). Blots were developed with antibodies against phosphor-Smad2/3 (top), Smad2/3 (middle), and β -actin (bottom). This experiment was repeated on three separate occasions, with similar results.

animal cell cultures were performed as described previously (13, 20, 29–35, 39–44). Pepsin–acid soluble collagen was measured by digesting the whole lung at 4°C with 0.1 mg/ml pepsin in 0.5 M acetic acid overnight, followed by a Sircol assay (Bicolor, Carrickfergus, UK), according to the manufacturer's recommendations. Total lung collagen was measured based on the quantification of hydroxyproline, using the colorimetric QuickZyme assay (QuickZyme BioSciences, Leiden, The Netherlands), according to the manufacturer's recommendations.

Statistical Analyses

Data are reported as means $\pm$ SDs. Differences between groups were evaluated with the Student two-tailed unequal-variance *t* test or the Mann-Whitney U test. *P* values less than 0.05 were considered statistically significant. To distinguish the synergistic (potentiating) and additive effects of the two treatments combined, the observed changes induced by the combined factors were compared with the sum of changes induced by each of the factors alone.

RESULTS

IL-33 Is Abundantly Expressed in the Lungs of Patients with ILD and in the Mouse Bleomycin Lung Injury Model

We previously reported that levels of IL-33 are elevated in patients with IPF (20). The association of elevated IL-33 with ILD was further extended because we observed a similar increase in IL-33–positive cells in patients with SLD (Figure 1A). An immunohistochemical evaluation of lung sections revealed that although some staining for IL-33 occurred in healthy lung control samples, the staining was substantially more intense in lung tissues from patients with IPF and SLD (Figure 1A). IL-33–expressing cells were abundant in the inflammatory infiltrates (*see* Figure E1 in the online supplement), which are known to consist mostly of lymphocytes, whereas only occasional macrophages, neutrophils, and eosinophils are present in the pulmonary infiltrates of these patients (7, 29, 31, 33, 35, 45). Although less intensely stained, stromal cells (likely to be fibroblasts, which are central to the pathogenesis of ILD) were also clearly positive for IL-33 (Figure 1A, with high magnifications on the *right*, in the *middle* and *bottom* rows). Of note, the IL-33 staining was predominantly intracellular and,

moreover, intranuclear. Cultured pulmonary fibroblasts expressed higher levels of IL-33 mRNA in patients with ILD, as measured by quantitative RT-PCR (Figure 1B). Considering the well-known activation and functional role of Smad2/3 signaling in ILD, primary normal human adult pulmonary fibroblast cultures were transfected by electroporation with human IL-33–encoding plasmid or, as a control, noncoding plasmid with the same backbone (both from Origene, Santa Cruz, CA), and IL-33 delivery was confirmed 72 hours later by quantitative RT-PCR (784 ± 16 –fold increase, triplicate cultures, two repeats) and ELISA of the cell lysates (21.8 ± 3.2 pg/ml in the IL-33–transfected cells, and undetectable in mock-transfected cells, triplicate cultures, two repeats). The lysates were analyzed by Western blotting with antibodies raised against phospho-Smad2/3 and unphosphorylated Smad2/3 (both from Santa Cruz Biotechnology, Santa Cruz, CA), revealing that IL-33 expression induces Smad2/3 phosphorylation (Figure 1C).

Subsequent experiments assessed the possibility that IL-33 may be similarly elevated in an animal model of ILD, specifically, in the bleomycin lung injury model. Similar to observations in human patients with ILD, C57BL/6 mice with bleomycin-induced lung disease also showed a substantial accumulation of IL-33–positive cells (Figures 2A–2D). To confirm the increases in IL-33 levels of lung tissue further, lung homogenates were analyzed in quantitative RT-PCR assays, using specific primers for IL-33 (Figure 2E). In parallel, ELISA assays with anti-IL-33 antibodies were performed (Figure 2F). These assays revealed significant increases in IL-33 mRNA and protein.

The observed intracellular, and predominantly intranuclear, localization of IL-33 suggested a lack of proteolytic maturation of this factor (20). Considering that mature IL-33 engages the T1/ST2 receptor, whereas the fl isoform does not (20), additional experiments assessed the potential involvement of T1/ST2 in bleomycin-challenged mice. No difference in total and differential cell counts was evident between T1/ST2-deficient and WT BALB/c mice (Figure E2A). Only occasional eosinophils (not exceeding 1% of total cells) were observed in both groups of mice, which is in contrast to the overt eosinophilia induced by mature but not

Figure 2. IL-33 mRNA and protein expression is elevated in mice challenged with intratracheal bleomycin (3–5 mice per group). Immunohistochemical staining reveals the expression of IL-33 in the lungs of mice challenged with bleomycin on Day 14. Sections were stained with either nonimmune (A) or anti-IL-33 (B–D) IgG, followed by counterstaining with hematoxylin (blue background staining in all sections). Cells expressing IL-33 are stained brown (aggregates of such cells are indicated with arrows in B and C, but not in D, to preserve clarity). In A and B, a $\times 10$ objective was used. The framed area in B was imaged with a $\times 20$ objective in C, and the framed area in C was

imaged with a $\times 40$ objective in D. These experiments were repeated in two independent experiments, using three mice in each experiment, with similar results. (E) Quantitative RT-PCR assays show a significant increase ($P < 0.05$) in IL-33 mRNA levels (fold change $\pm$ SD) in bleomycin-treated mice (BLM) compared with control saline-treated mice (PBS) on Days 7 (7 d) and 14 (14 d). (F) ELISA assays of lung homogenates from mice challenged with PBS or bleomycin show significant increases in IL-33 levels ($P < 0.05$) on Days 7 and 14, as indicated.

flIL-33 (20). These findings jointly suggested that full-length rather than mature IL-33 is involved in ILD.

IL-33 Potentiates Bleomycin-Induced Lung Injury *In Vivo*

Subsequent experiments addressed the possibility that elevated IL-33 expression may contribute to bleomycin-induced lung injury. WT C57Bl/6 mice were first infected by intratracheal instillations with either flmIL-33–encoding adenovirus (AdV-flmIL33), as described previously (20), or with control adenovirus not encoding a cytokine (AdV-NULL) and then challenged with bleomycin or PBS 7 days later. Similar adenoviral loads in all experimental groups were confirmed by ELISA measurements of the pulmonary levels of enhanced green fluorescent protein (EGFP), which was encoded in the backbone of all constructs under control of a separate promoter. The levels of EGFP were consistently measured at between 1.7 and 2.3 ng/ml on Day 14 after intratracheal instillations, as previously reported in this model, but undetectable in control mice; please refer to Figure 2C in Luzina and colleagues (20). The expression levels of IL-33 protein in lung homogenates were tested by ELISA, and were consistently between 40 and 50 ng/ml on Day 14 after intratracheal instillations, as previously reported in this model; please see Figure 2D in Luzina and colleagues (20). These levels of adenovirally delivered IL-33 are comparable to those independently induced by bleomycin challenge (Figure 2F). BAL samples and lung tissues were harvested at later time points and analyzed, with results to be described. Differential BAL cell counts revealed that IL-33 expression exerted a synergistic (potentiating) effect on the BAL lymphocytosis induced by bleomycin, with the effect of the combination significantly greater than that of the sum of the independent effects (Figure 3A). In contrast, no synergy was observed on BAL macrophages and the effect on BAL neutrophils was additive, that is, the effect of IL-33 expression and bleomycin combined did not significantly exceed the sum of their independent effects (Figure E3). Again, only occasional BAL eosinophils (not exceeding 1% of total cells) were observed in all of these mice. Flow-cytometric analyses revealed that T lymphocytes, including CD4⁺ and CD8⁺ cells, as well as B lymphocytes were accumulating in the lungs of mice, and that the fraction of CD19⁺ cells was further elevated in mice challenged with IL-33 expression and bleomycin combined (Figure E4). The hematoxylin-and-eosin staining of lung tissues revealed more severe inflammatory infiltrates in mice challenged with IL-33 expression and bleomycin injury than in mice challenged with each of these factors alone (Figure 3B).

Bleomycin injury induces an excess deposition of extracellular matrix in mouse lungs, modeling pulmonary fibrosis in humans. Assays assessed the combined effects of IL-33 expression and bleomycin injury on pulmonary collagen in experimental mice (Figure 4). The results for trichrome staining of lung sections suggested that, although IL-33 expression alone and bleomycin injury alone induced collagen deposition, this effect was more pronounced when these two factors were combined (Figure 4A). Initial quantitative tests assessed the accumulation of pepsin–acid soluble collagen, using a Sircol assay. The results suggested that IL-33 gene delivery elevated total lung collagen from 47.7 ± 5.8 $\mu\text{g/lung}$ in AdV-NULL–challenged mice to 94.9 ± 6.2 $\mu\text{g/lung}$ in AdV-flmIL-33–challenged mice, 14 days after gene delivery. These tests were limited because (1) they measured only pepsin–acid soluble and not total lung collagen, and (2) Sircol assays are flawed by the interference of noncollagenous proteins, for example, those that accumulate in the lung as part of the inflammatory process (46). To assess total levels of pulmonary collagen more precisely, hydroxyproline-based QuickZyme tests were used, which include complete tissue hydrolysis in 6 M HCl at 95°C, followed by the quantitative determination of collagen, based

Figure 3. Synergistic effects of full-length mouse IL-33 (flmIL33) gene delivery *in vivo* on BLM-induced lung injury in mice (3–5 mice per group per time point, repeated on two separate occasions with similar results). (A) Bronchoalveolar lavage (BAL) lymphocyte counts (mean $\pm$ SD) in mice challenged with combinations of control adenovirus not encoding a cytokine (AdV-NULL) or IL-33–encoding adenovirus (AdV-IL-33) infection and BLM or PBS instillations, as indicated. The intratracheal instillation of the AdV took place 7 days before the BLM or PBS challenge, and the time points are shown as the number of days after NULL, PBS, or IL-33 delivery/number of days after BLM or PBS challenge. Differences in lymphocyte counts were evaluated using the two-tailed *t* test. Differences from NULL + PBS were significant ($P < 0.05$) in all other groups at all tested times, but are not marked as significant to preserve clarity. To confirm synergy, the observed response to combined IL-33 expression and BLM challenge was compared with the response to be expected based on the additive action of IL-33 expression alone and BLM alone. An additive effect is indicated with a *single asterisk*, and a synergistic effect with *stacked asterisks*. (B) Hematoxylin-and-eosin staining of lung sections from mice challenged with the same combinations of gene delivery and bleomycin injury, Days 21/14, $\times 20$ objective.

Figure 4. Effects of IL-33 gene delivery and bleomycin injury on pulmonary collagen *in vivo*, on Days 21/14 after AdV instillations/BLM challenge, respectively (3–5 animals per group, two or three separate experiments with similar results in all cases). (A) Trichrome staining of lung sections shows collagen fibers in blue (selected areas are indicated with arrowheads; $\times 40$ objective). (B) Total lung collagen, based on hydroxyproline measurements in QuickZyme assays (QuickZyme BioSciences). (C) Steady-state mRNA levels were measured by quantitative RT-PCR for $\alpha 1(I)$ (COL1A1) and $\alpha 1(III)$ (COL3A1) collagen chains. *Synergistic effects.

on hydroxyproline levels in the lysate. These assays are calibrated with known amounts of collagen standard, rather than the free amino acid hydroxyproline. Quantitative measurements of total lung collagen, based on the levels of pulmonary hydroxyproline, showed that, indeed, combined IL-33 expression and bleomycin injury led to the accumulation of more collagen than did either of these two factors alone (Figure 4B). The results of combined IL-33 expression and bleomycin injury indicated an additive rather than a synergistic effect on collagen deposition (Figure 4B). Notably, the effects of bleomycin on collagen accumulation in the lungs were independent of the T1/ST2 receptor (Figure E2B). Quantitative RT-PCR assays showed that the gene delivery of IL-33 exerted a synergistic effect on the steady-state levels of collagen $\alpha 1(I)$ (COL1A1) and COL3A1 mRNA induced by bleomycin (Figure 4C). Thus, the expression of flIL-33 worsens the effects of bleomycin injury on pulmonary inflammation (Figure 3) and fibrosis (Figure 4).

Synergistic Cytokine Regulation by flIL-33 and Bleomycin Injury

We considered the possibility that the observed synergy between flIL-33 expression and bleomycin injury was mediated by their combined effect on cytokine expression. Multiplex assays were used to measure cytokine levels, and ELISA was used to measure transforming growth factor- β (TGF- β) in the pulmonary homogenates of experimental mice. The levels of several important cytokines (total TGF- β , monocyte chemoattractant protein-1 [MCP-1], macrophage inflammatory protein-1 α [MIP-1 α], IL-6, and tumor necrosis factor- α [TNF- α]) were synergistically induced by IL-33 expression and bleomycin injury (Figure 5). All

these cytokines are known to regulate inflammation and fibrosis centrally, as reviewed elsewhere (4, 6, 7). Furthermore, these observations are consistent with a previous report on the stimulating effect of flIL-33 on the levels of IL-6 and MCP-1 mRNAs (20). Thus, the synergistic action of flIL-33 expression and bleomycin injury (Figures 3 and 4) is likely mediated by these cytokines (Figure 5). By contrast, the bleomycin-induced moderate increases in murine IL-8 homologue KC and IL-1 β were not significantly different from those observed in IL-33-expressing mice challenged with bleomycin ($P > 0.05$). Of note, the levels of total TGF- β were, whereas the levels of naturally active TGF- β were not synergistically up-regulated by flIL-33 expression and bleomycin injury (Figure 5). This discrepancy may be explained by the known association of active TGF- β with the extracellular matrix and cell surface *in vivo*, leading to its rapid consumption by cells (35, 47). Without acid activation, which is used for measuring total TGF- β by ELISA, the majority of naturally active TGF- β may remain in the insoluble fraction of pulmonary homogenate samples. No changes in the expression of IL-4 or IL-13 were evident, and the overall levels of these central Th2 cytokines were at or below the detection threshold in all samples, which is in contrast with their elevation in mature IL-33-expressing mice (20) or ovalbumin-challenged mice (40, 41). This observation is in agreement with previous data showing that flIL-33 is not a pro-Th2 cytokine (20). Furthermore, this finding suggests that although Th2 cytokines are clearly profibrotic, as reviewed elsewhere (4, 6, 7), this mechanism is unlikely to contribute to the observed synergy between flIL-33 expression and bleomycin in pulmonary changes of cytokine levels.

Figure 5. ELISA for transforming growth factor-β (TGF-β) and multiplex measurements of IL-6, monocyte chemotactic protein-1 (MCP-1; CCL2), and macrophage inflammatory protein-1α (MIP-1α; CCL3) in lung homogenates. Data are presented as pg/ml, mean ± SD, 3–5 mice per group for each time point (days after Adv instillations/BLM challenge). To confirm synergy, the observed response to the combined IL-33 expression and BLM challenge was compared with the response expected based on the additive action of IL-33 expression alone and BLM alone. An additive effect is indicated with a *single asterisk*, and a synergistic effect with *stacked asterisks*. To be designated as synergistic, the significance of the difference between the observed combined effect and the expected additive effect had to be less than 0.05.

Elevation of Pulmonary HSP70

To further address the possible mechanisms involved in the combined effects of flIL-33 gene delivery and bleomycin injury, gene expression profiling pilot experiments using Affymetrix Mouse Genome 430 version 2.0 (Santa Clara, CA) short oligomer arrays were performed in TC-1 cells infected with Adv-flmIL-33, Adv-mmIL-33, or Adv-NULL. The results suggested that flmIL-33 up-regulated the expression of HSP70-1 (encoded by Hspa1a), HSP70-2 (encoded by Hspa1b), HSP27 (encoded by Hspb1), and HSP110 (encoded by Hspa4) mRNAs. To assess the magnitude of these changes more precisely, quantitative RT-PCR experiments were performed on TC-1 cells and primary normal mouse lung fibroblast cultures similarly infected with Adv. Quantitative results revealed that the expression of these genes was increased by the gene delivery of either flmIL-33 or mmIL-33 (Figures 6A and 6B). Importantly, the induced

changes in HSP70-1 and HSP70-2 were more pronounced for the flmIL-33 than for the mmIL-33 delivery (Figures 6A and 6B). Subsequent immunohistochemical analyses of lung sections (Figure 6C) revealed the moderate expression of HSP70, predominantly in bronchial epithelium. The epithelial expression of HSP70 was increased in IL-33-expressing and bleomycin-challenged mice, but in these cases, abundant HSP70-positive infiltrating cells were also evident (Figure 6C). This HSP70-positive result was particularly pronounced in mice challenged with combined IL-33 expression and bleomycin, with a massive presence of infiltrating and epithelial cells expressing HSP70, whereas the infiltrating HSP70-positive cells appeared in clusters (arrows in Figure 6C, lower right), as well as scattered through the lung parenchyma. Furthermore, ELISAs of BAL fluids for extracellular HSP70 showed increases after all treatments, with a synergistic effect after IL-33 delivery and bleomycin challenge on Days

Figure 6. Changes in the expression of heat shock protein (HSP) induced by IL-33. (A and B) Quantitative RT-PCR analyses of TC-1 cell cultures (A) and normal mouse lung fibroblast cultures (B) infected with full-length mouse IL-33 (AdV-flmIL-33) or mature mouse IL-33 (AdV-mmIL-33), as indicated, in comparison with empty vector (AdV-NULL)-infected cells. Mean fold increase $\pm$ SD in the levels of indicated mRNAs are indicated, 48 hours after infection. Note the log scale on the vertical axis. The assay was repeated on three occasions with similar results. (C) Immunohistochemistry for HSP70, using an antibody reactive to both HSP70-1 and HSP70-2 (Abcam, Cambridge, MA) in mice challenged with the indicated factors. Tissues were obtained 21 days after AdV-NULL or AdV-IL-33 instillation, and 14 days after BLM or PBS challenge. HSP70-expressing cells are stained brown in epithelium and infiltrating cells (selective areas are indicated with arrows). Treatment with NULL + BLM resulted in an HSP70 elevation that was indistinguishable from that induced by PBS + BLM. The assay was repeated for three mice in each group, with similar results. (D) ELISA of BAL fluids for extracellular HSP70 (mean pg/ml $\pm$ SD, 3–5 mice per group for each time point, days after AdV instillation/BLM challenge).

21 and 14, respectively (Figure 6D). Thus, IL-33 and bleomycin injury cooperate in elevating the levels of both intracellular and extracellular HSP70.

DISCUSSION

Our results show that IL-33-expressing cells are abundant in the lungs of patients with IPF and SLD (Figure 1). Mice challenged with bleomycin respond by a similar elevation of pulmonary IL-33

expression (Figure 2). We previously found that the main portion of IL-33 in the lungs of patients is not proteolytically processed, that is, it is fl (20). In the combined flIL-33 expression and bleomycin challenge model, synergistic effects were observed on lung inflammation, particularly in terms of pulmonary lymphocytosis (Figure 3), and on collagen accumulation (Figure 4). Such potentiation could be explained by the synergistic regulation of the cytokines TGF- β , IL-6, MCP-1, MIP-1 α , and TNF- α (Figure 5), and was associated with elevated HSP70 (Figure 6).

These effects were specifically induced by the delivered fIL-33, and were not artifacts induced by the adenoviral vehicle or nonspecific consequences of accumulations of the overexpressed proteins in pulmonary cells. First, mice instilled with AdV-NULL showed minimal, if any, infiltration of cells in the lungs, suggesting that the response to the infection with the nonreplicating AdV was minimal within the timeframe of the experiments. Second, all adenoviral constructs encode a foreign protein (EGFP) in their backbone under a separate promoter, leading to strong EGFP expression in cells infected with any of these viruses. AdV-NULL-infected mice overexpressing EGFP in their lungs show minimal if any pulmonary response, suggesting that the overexpression of a foreign protein exerts a minimal effect on the lung within the experimental timeframe. Third, we made a similar unpublished observation using the recombinant AdV-mediated delivery of an unrelated protein, keratoepithelin, in which the expression in mouse lungs did not cause the pulmonary infiltration of inflammatory cells or changes in the cytokine profile. Fourth, in contrast to the observed effects of fIL-33, a similar recombinant AdV-mediated delivery of IL-4 isoforms (40, 41) or CCL18 (13, 35, 39) resulted in different changes in BAL cellularity and the pulmonary cytokine milieu, suggesting that the effects on the lung are specific to the delivered factor. Finally, adenoviral vectors have undergone significant investigation in the clinic, and showed immense promise for the delivery of desired genes (48). Taken together, these observations suggest that the observed outcomes are specific to the proteins being delivered, and are different from an overall inflammatory response, cell injury, or broad immune activation.

These observations suggest that fIL-33 is not only an independent regulator of lung inflammation (20), but also that it enhances the proinflammatory and profibrotic injury induced by an independent insult, such as bleomycin. A possibility exists that the delivered fIL-33 undergoes proteolytic maturation in the lungs of mice with or without bleomycin challenge, although we have already shown, based on the difference in molecular weights between the fl and mature IL-33 forms exhibited in Western blotting, that such spontaneous maturation upon AdV-mediated fIL-33 delivery does not occur in cell cultures (20). Unfortunately, none of the commercial anti-IL-33 antibodies that were tested allowed for a similar direct demonstration in lung tissue because of nonspecific binding to the molecular components of the lung across a broad range of molecular weights. Furthermore, the levels of IL-33 isoforms in BAL fluid were too low to be analyzed reliably by immunoblotting. However, evidence suggests that the delivered fIL-33 does not undergo proteolytic maturation in the lungs *in vivo*, with or without bleomycin challenge. First, mature IL-33 is well known to induce eosinophilia, goblet-cell hyperplasia, and an elevation in the expression of Th2 cytokines (18–20), yet no such changes were observed in these experiments. Second, mature IL-33 acts through the T1/ST2 receptor (18–20), but a germline deficiency of T1/ST2 did not affect the ability of fIL-33 to induce pulmonary inflammation (20), and exerted no effect on bleomycin-induced inflammation or fibrosis (Figure E2), further indicating that mature IL-33 was unlikely to be involved in the observed pulmonary changes. Several groups have reported that fIL-33 released from damaged cells may bind to the T1/ST2 receptor, activate NF- κ B, and increase the production of IL-6 in cell cultures (49–51). However, in the absence of overt cell damage *in vivo*, elevated fIL-33 remains predominantly intranuclear, and acts by regulating gene expression rather than through binding to T1/ST2 (20).

In the present study, the synergistic effects of fIL-33 and bleomycin challenge *in vivo* were observed in the expression of key cytokines that are known to propel pulmonary fibrosis.

TGF- β is a central regulator of inflammation and fibrosis (52), and IL-6 (53) and the chemokines MCP-1 and MIP-1 α contribute significantly to these processes (6, 54). Additional scanning of the fIL-33-mediated regulation of gene expression in cell culture revealed a previously unknown effect on the expression of HSP, and particularly HSP70 (Figure 6A). Similar to a significant increase in HSP70 in human patients with progressive IPF (25), the presence of HSP70-positive cells was particularly pronounced in the combined fIL-33 delivery and bleomycin injury model. It remains unclear whether HSP acts profibrotically (27, 28) or antifibrotically (9–12), but its possible protective effect was not sufficient to overcome the potent cytokine-driven changes in the lungs in this model. According to one possibility, intracellular HSP70 and extracellular HSP70 differentially regulate ILD, with the intracellular form being cytoprotective and the extracellular form acting as a proinflammatory and profibrotic mediator, particularly through the Toll-like receptor-2 (TLR2) and TLR4 pathways (28, 55–60), which regulate responses to bleomycin injury (61–64). In our experiments, IL-33 and bleomycin challenge cooperatively activated the levels of both intracellular (Figure 6C) and extracellular (Figure 6D) HSP70. Further studies are needed to outline the exact mechanistic links between fIL-33, HSP70, TLR signaling, and proinflammatory and profibrotic responses in ILD.

In conclusion, bleomycin-induced lung injury is potentiated by fIL-33 in a T1/ST2-independent, Th2-independent fashion, likely through cytokine regulation and possibly by engaging HSP70. These findings suggest that the cell-surface receptor-independent regulation of gene expression by fIL-33 may critically contribute to the pathogenesis of ILD and, perhaps, other pathologies characterized by the elevation of fIL-33 expression.

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Dr. Edward Pickering for help with some of the experiments, and Dr. Paul Todd for expert editorial help.

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