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IRAK-M Promotes Alternative Macrophage Activation and Fibroproliferation in Bleomycin-Induced Lung Injury

Megan N. Ballinger,* Michael W. Newstead,† Xianying Zeng,† Urvashi Bhan,† Xiaokui M. Mo,‡ Steven L. Kunkel,§ Bethany B. Moore,† Richard Flavell,¶ John W. Christman,* and Theodore J. Standiford†

Idiopathic pulmonary fibrosis is a devastating lung disease characterized by inflammation and the development of excessive extracellular matrix deposition. Currently, there are only limited therapeutic intervenes to offer patients diagnosed with pulmonary fibrosis. Although previous studies focused on structural cells in promoting fibrosis, our study assessed the contribution of macrophages. Recently, TLR signaling has been identified as a regulator of pulmonary fibrosis. IL-1R–associated kinase-M (IRAK-M), a MyD88-dependent inhibitor of TLR signaling, suppresses deleterious inflammation, but may paradoxically promote fibrogenesis. Mice deficient in IRAK-M (IRAK-M<sup>−/−</sup>) were protected against bleomycin-induced fibrosis and displayed diminished collagen deposition in association with reduced production of IL-13 compared with wild-type (WT) control mice. Bone marrow chimera experiments indicated that IRAK-M expression by bone marrow–derived cells, rather than structural cells, promoted fibrosis. After bleomycin, WT macrophages displayed an alternatively activated phenotype, whereas IRAK-M<sup>−/−</sup> macrophages displayed higher expression of classically activated macrophage markers. Using an in vitro coculture system, macrophages isolated from in vivo bleomycin-challenged WT, but not IRAK-M<sup>−/−</sup>, mice promoted increased collagen and α-smooth muscle actin expression from lung fibroblasts in an IL-13–dependent fashion. Finally, IRAK-M expression is upregulated in peripheral blood cells from idiopathic pulmonary fibrosis patients and correlated with markers of alternative macrophage activation. These data indicate expression of IRAK-M skew lung macrophages toward an alternatively activated profibrotic phenotype, which promotes collagen production, leading to the progression of experimental pulmonary fibrosis. 


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Abbreviations used in this article: AEC, alveolar epithelial cell; BAL, bronchoalveolar lavage; BALF, BAL fluid; BMDM, bone marrow–derived macrophage; ILC2, type 2 innate lymphoid cell; INOS, inducible NO synthase; IPF, idiopathic pulmonary fibrosis; IRAK-M, IL-1R–associated kinase-M; IL-13, interleukin-13; mRNA, murine mRNA; rIL-13, α-SMA, alpha smooth muscle actin; WT, wild-type.

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that IL-13 can also drive this phenotypic switch. Incubation of human lung fibroblasts with conditioned media from IFP macrophages induces procollagen expression from fibroblasts (12), demonstrating regulation of extracellular matrix deposition by alternatively activated macrophages.

TLRs recognize evolutionarily conserved motifs important in inflammation and immunity. Although originally defined as pathogen recognition receptors, TLRs also recognize endogenously produced molecules (13). Recent work has begun to address the role of TLRs in pulmonary fibrosis. The combined absence of both TLR2 and TLR4 increased susceptibility to bleomycin- and radiation-induced pulmonary fibrosis (14, 15). Moreover, MyD88−/− mice are more susceptible to pulmonary fibrosis, as compared with wild-type (WT) mice (16). Conversely, TLR2 was found to be elevated in IPF patients (17), and the pharmacologic inhibition of TLR2 protected mice against bleomycin challenge (18). IL-1R–associated kinase-M (IRAK-M or IRAK-3) is a negative regulator of MyD88-dependent TLR signaling. Originally described in monocytes and macrophages, IRAK-M has also been found in epithelial cells and cardiac fibroblasts (19–21). Expression of IRAK-M is upregulated by profibrotic molecules, such as IL-13 (22) and TGF-β (23). Additionally, this molecule has been shown to suppress deleterious inflammation in influenza-induced lung injury, but mediates impaired antibacterial immunity in the setting of experimental sepsis (24, 25). Recently, our laboratory has shown that IRAK-M−/− mice are protected from hyperoxic lung injury (26), and the absence of IRAK-M skews tumor-associated macrophages toward classical rather than alternative activation (23). However, the role of IRAK-M in regulating macrophage activation in lung fibroproliferative responses has not been examined. Our data show that IRAK-M promotes alternative macrophage activation in the setting of bleomycin-induced lung injury, which drives lung fibrogenesis in an IL-13–dependent fashion.

Materials and Methods

Animals

A colony of IRAK-M−/− (deficient) mice bred on a B6 background for >10 backcrosses was established at the University of Michigan (24). Age- and sex-matched specific pathogen-free 6- to 8-wk-old C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The animals were housed in specific pathogen-free conditions within the University of Michigan Animal Care Facility (Ann Arbor, MI). All animal experiments proceeded in accordance with National Institutes of Health policies on the human care and use of laboratory animals and were approved by the University Committee on Use and Care of Animals at the University of Michigan.

Bleomycin-induced pulmonary fibrosis and histology

WT and IRAK-M−/− mice were injected with bleomycin (0.025 U; Sigma-Aldrich) intratracheally (i.t.) on day 0. Lungs were collected on days 0, 7, 14, and 21 to measure cytokine protein expression by ELISA and mRNA expression by real-time RT-PCR and on days 14 and 21 for histology and determination of collagen content by hydroxyproline assay (27). For histology samples, lungs were perfused with saline and inflated with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO). Sections were stained with H&E and Masson’s trichrome.

Bronchoalveolar lavage

Mice were euthanized and BAL was performed, as previously described (28). Lavaged cells from each group of animals were pooled, and cytocinsps (Thermo Electron, Waltham, MA) were prepared for determination of BAL differentials using a modified Wright-Giemsa stain.

ELISA

The concentration of albumin was determined via mouse albumin ELISA quantification kit (Bethyl Laboratories, Montgomery, TX). The amount of cytokines and chemokines in the BAL, lung homogenates, and lung macrophages was determined via DouSet ELISA (R&D Systems, Minneapolis, MN). Specifically, the amount of TGF-β production was measured in acid-activated supernatants to measure total active TGF-β.

Isolation of macrophages

Macrophages were isolated from the whole lungs of mice via a collagenase digestion, as previously described (24). Cells were adherence purified for 1 h in serum-free media; nonadherent cells (such as lymphocytes) were washed away; and complete media was replaced for overnight incubation. After overnight incubation, macrophage purity was >95%, as determined by modified Wright-Giemsa stain. Bone marrow cells were isolated from the femurs of WT and IRAK-M−/− mice and differentiated into mature macrophages in DMEM supplemented with 10% FBS, 50 IU/ml penicillin, and 50 μg/ml streptomycin, and 20 ng/ml mouse rM-CSF added on days 1 and 4. Cells were allowed to differentiate up to 7 d in total. Macrophages were stimulated overnight with either murine rIL (rmIL)-13 (10 ng/ml) and/or rmIL-13 and rmIL-4 (10 ng/ml) (R&D Systems).

Murine alveolar epithelial cell isolation and cell culture conditions

Murine type II alveolar epithelial cells (AECs) were isolated using the method developed by Corti et al. (29). Briefly, the pulmonary vasculature was perfused, and the lungs were filled with 1 ml dispoase (Worthington, Lakewood, NJ) and then 1 ml low-melting-point agarose, and finally placed in ice-cold PBS to harden. The lungs were then submerged in dispase for 45 min before being minced and incubated in DMEM with 0.01% DNase for 10 min. A single-cell suspension was obtained by passing the lung mince over a series of nylon filters. Myeloid cells were removed by first incubating cells with biotinylated Abs against CD32 and CD45 (BD Pharmingen, San Diego, CA) and then streptavidin-coated microbeads (Promega, Madison, WI), and performing a negative selection using a magnetic tube separator. Mesenchymal cells were removed by
overnight adherence in a petri dish, and the resulting nonadherent AECs were either assayed immediately (ex vivo AECs) or plated on fibronectin-coated dishes for designated time periods. Previous work has shown that the day 3 time point has >90% pure AECs (29).

**Bone marrow transplantation**

WT and IRAK-M<sup>−/−</sup> mice received 13 Gy total body irradiation (137Cs source) delivered in two fractions separated by 3 h. Bone marrow was harvested from the femurs of donor mice, and BM was transplanted into the lethally irradiated recipients via tail vein infusion. All experiments with harvested from the femurs of donor mice, and BM was transplanted into marrow transplantation mice were performed 6–8 wk after bone marrow transplantation, as previously described (30).

**Semiquantitative real-time RT-PCR**

Real-time RT-PCR was performed on an ABI Prism 7000 thermocycler (Applied Biosystems, Foster City, CA) and analyzed as previously described (26). Gene-specific primers and probes were purchased from Integrated DNA Technologies (Coralville, IA). 

**Western blotting**

Lung macrophages were lysed in buffer containing radioimmunoprecipitation assay (Sigma-Aldrich) supplemented with protease inhibitors (Roche Diagnostics), and gels were subjected to electrophoresis, as previously described (26). Membranes were incubated with Abs against IRAK-M (Abcam; 1:1,000), total STAT6 (Cell Signaling; 1:500), phosphor-STAT6 (Abcam; 1:500), or β-actin (Abcam; 1:10,000). Signals were developed with an ECL Plus Western blot detection kit (Amersham, Arlington Heights, IL). Densitometry was calculated using Image J.

**Macrophage or macrophage supernatant and fibroblast coculture**

Fibroblasts were isolated from WT unchallenged mice, as previously described (4). After 14 d in culture, lung fibroblasts were removed, counted, plated at 5 × 10<sup>4</sup> cells/well, and serum starved overnight. Lung leukocytes were isolated from either unchallenged or day 14 bleomycin-challenged WT and IRAK-M<sup>−/−</sup> mice. CD45<sup>+</sup> cells were isolated from the total lung leukocytes by SuperMacs column (Miltenyi Biotec, Auburn, CA). Total cells were enumerated by hemocytometer, and percentage of macrophages was assessed by hypdroxyproline assay (21 and amount of collagen was as-

**Results**

Bleomycin induces expression of IRAK-M in lung macrophages and AECs

To determine whether IRAK-M expression was upregulated during experimental fibrosis, mRNA and protein expression of IRAK-M was >95%, as determined by histological staining. Additionally, supernatants were isolated from ∼1 × 10<sup>6</sup> macrophages and spun down to remove all cellular debris before being added to WT fibroblast cultures. Finally, either control IgG or anti–IL-13 neutralizing Ab (2 μg/ml; provided by S. Kunkel, University of Michigan) was added to the coculture systems. Cocultures were then incubated at 37˚C for 48 h before isolating mRNA by TRIzol extraction.

**Gene profiling for peripheral blood RNA of IPF and healthy subjects**

The data presented in this study were generated using a search of the Gene Expression Omnibus Profiles database [data accessible at National Center for Biotechnology Information Gene Expression Omnibus database GSE33566 (31)]. Peripheral blood gene expression data (collected in PAXgene RNA tubes, PreAnalytiX, 762164; Qiagen) from 93 IPF samples and 30 healthy normal controls were analyzed. IPF subjects had a consensus diagnosis of probable or definite IPF based on the American Thoracic Society /European Respiratory Society/Japanese Respiratory Society/Latin American Thoracic Association criteria and pulmonary function measurements (lung diffusion capacity or forced vital capacity) (31). Subjects were excluded from selection if they were current smokers, or currently treated with agents that could alter mRNA levels such as glucocorticoids, azathioprine, or other immunomodulators. Data were normalized in Expression Console (Affymetrix, Santa Clara, CA) using robust multiaarray average method. Two sample t tests were used to compare the difference in gene expression levels in peripheral blood RNA between IPF patients and healthy donors. The correlation between IRAK-M and arginase was assessed by Pearson correlation method.

**Statistical analysis**

All data are expressed as mean ± SEM and were analyzed using Prism 5.0 statistical program (GraphPad Software). Comparisons between two experimental groups were performed with Student’s t test. Comparisons among three or more experimental groups were performed with ANOVA with a post hoc Bonferroni test to determine significance. A p value <0.05 was considered significant.
was measured in isolated lung cells after bleomycin challenge. There was a marked time-dependent increase in the expression of IRAK-M mRNA (Fig. 1A) and protein (Fig. 1B) beginning at 7 d (>12-fold increase in protein) after bleomycin challenge and continuing through day 14 d in pulmonary macrophages isolated from dispersed lung. We also observed an early increase in the expression of IRAK-M mRNA in AECs isolated from bleomycin-challenged mice at day 3 (maximal 4.5-fold increase), before returning to baseline by day 7 (Supplemental Fig. 1A). No induction of IRAK-M was noted in lung fibroblasts isolated from bleomycin-treated mice at any time points after challenge (Supplemental Fig. 1B). These data demonstrated a substantial induction of IRAK-M expression in lung macrophages and to a lesser extent AEC after bleomycin administration.

**IRAK-M regulates bleomycin-induced lung fibrosis**

Modulation of IRAK-M expression has previously correlated with altered susceptibility to injury and infection (24–26); however, the role of this negative TLR regulator in pulmonary fibrosis has yet to be explored. To determine whether IRAK-M was critical for the development of bleomycin-induced pulmonary fibrosis, WT and IRAK-M<sup>-/-</sup> mice were challenged with bleomycin and the amount of fibrosis was measured. To quantitate fibrosis, whole lungs were harvested from WT and IRAK-M<sup>-/-</sup> at days 14 and 21 after bleomycin and the amount of collagen was measured by hydroxyproline assay. As compared with unchallenged control mice, WT mice had significantly higher collagen deposition at days 14 and 21 after bleomycin challenge. In contrast, there was no significant increase in collagen deposition in whole lungs from IRAK-M<sup>-/-</sup> mice after bleomycin administration (Fig. 2A). We also observed substantially greater induction of collagen III mRNA in whole-lung samples from WT mice at 14 d after bleomycin (4.5-fold increase compared with 1.4-fold increase) as compared with IRAK-M<sup>-/-</sup> mice (Fig. 2B). Finally, increased collagen deposition as shown by enhanced trichrome staining was observed on both days 14 and 21 in the WT mice challenged with bleomycin. By comparison, there was a relatively modest increase in collagen deposition and preserved lung architecture in IRAK-M<sup>-/-</sup> mice after administration of bleomycin (Fig. 2C). These data show that IRAK-M<sup>-/-</sup> mice were protected from bleomycin-induced pulmonary fibrosis, indicating that IRAK-M is a key mediator of fibrogenesis in response to bleomycin.

**Alterations in leukocyte influx and alveolar permeability in IRAK-M<sup>-/-</sup> mice after bleomycin challenge**

To determine whether IRAK-M regulated inflammatory cell recruitment after bleomycin challenge, the total number and subtype of leukocytes were enumerated from whole lung digestion. Administration of bleomycin to WT mice resulted in an increase in lung neutrophils at both 3 and 7 d after treatment (Fig. 3A). As compared with WT mice, there was a significant increase in neutrophils in IRAK-M-deficient mice at 3 d after challenge with bleomycin, whereas by 7 d neutrophil numbers were less than that observed in WT controls (Fig. 3A). Conversely, an increase in lung macrophages was not observed until 7 d after bleomycin in WT mice, whereas no significant increase was noted in IRAK-M<sup>-/-</sup> mice at either 3 or 7 d (Fig. 3B). However, we observed no difference in the production of chemokines, including CXCL1/KC, CXCL2/MIP-2α, or CCL2/MCP-1, or GM-CSF between WT and IRAK-M<sup>-/-</sup> mice after bleomycin challenge (data not shown).

To determine whether IRAK-M expression regulated lung permeability after bleomycin challenge, the amount of albumin in the BALF was measured as a surrogate for alveolar permeability. In WT mice, treatment with bleomycin resulted in a time-dependent increase in BAL albumin, maximum 7 d after bleomycin (Fig. 3C). Interestingly, albumin levels were significantly less in IRAK-M<sup>-/-</sup> mice 14 d after bleomycin challenge as compared with their WT counterparts.

**IRAK-M expression by bone marrow cells rather than structural cells enhances susceptibility to bleomycin-induced pulmonary fibrosis**

The relative contribution of IRAK-M expression in structural cells and bone marrow–derived cells after bleomycin-induced lung injury was delineated by generating bone marrow chimeras. Bone marrow chimeras were generated by mixing bone marrow cells from WT and IRAK-M<sup>-/-</sup> mice 30 days before administration of bleomycin. WT and IRAK-M<sup>-/-</sup> mice were challenged with bleomycin and the amount of collagen was measured by hydroxyproline assay. As compared with unchallenged control mice, WT mice had significantly higher collagen deposition at days 14 and 21 after bleomycin challenge. In contrast, there was no significant increase in collagen deposition in whole lungs from IRAK-M<sup>-/-</sup> mice after bleomycin administration (Fig. 2A). We also observed substantially greater induction of collagen III mRNA in whole-lung samples from WT mice at 14 d after bleomycin (4.5-fold increase compared with 1.4-fold increase) as compared with IRAK-M<sup>-/-</sup> mice (Fig. 2B). Finally, increased collagen deposition as shown by enhanced trichrome staining was observed on both days 14 and 21 in the WT mice challenged with bleomycin. By comparison, there was a relatively modest increase in collagen deposition and preserved lung architecture in IRAK-M<sup>-/-</sup> mice after administration of bleomycin (Fig. 2C). These data show that IRAK-M<sup>-/-</sup> mice were protected from bleomycin-induced pulmonary fibrosis, indicating that IRAK-M is a key mediator of fibrogenesis in response to bleomycin.

**FIGURE 3.** IRAK-M reduces alveolar permeability and cellular recruitment after bleomycin challenge. WT and IRAK-M<sup>-/-</sup> mice were given an i.t. challenge with bleomycin. (A and B) BAL were performed on WT and IRAK-M<sup>-/-</sup> mice at days 0, 3, and 7 after bleomycin challenge, and the total numbers of neutrophils or mononuclear cells were quantified. (C) The amount of albumin in the BALF was quantified as a surrogate for the amount of lung leakage after bleomycin challenge. Graphs represent mean ± SEM with n = 3 samples/group from three separate experiments. *p < 0.05, **p < 0.01, ***p < 0.001 when compared with day 0 WT control sample, and ×p < 0.05 when compared with day 0 IRAK-M<sup>-/-</sup> control sample.
marrow was harvested from WT or IRAK-M$^{-/-}$ mice and transplanted into either IRAK-M$^{-/-}$ or WT lethally irradiated recipients, as described in Materials and Methods. Five weeks after reconstitution of bone marrow cells, mice were administered an i.t. challenge with bleomycin. Whole lung was collected from either unchallenged or bleomycin-challenged mice at day 14 or 21, and the amount of fibrosis was determined by hydroxyproline assay and trichrome staining of histological samples. In bleomycin-challenged animals, there was a significant increase in the amount of hydroxyproline in the WT$\rightarrow$WT group (2.5-fold increase) and the WT$\rightarrow$IRAK-M$^{-/-}$ group (2.21-fold increase) (Fig. 4A). As anticipated, we observed no increase in hydroxyproline levels in the control IRAK-M$^{-/-}$$\rightarrow$IRAK-M$^{-/-}$ after bleomycin challenge. Interestingly, when IRAK-M was absent in the bone marrow compartment (IRAK-M$^{-/-}$$\rightarrow$WT group), there was also no increase in hydroxyproline levels (Fig. 4A). To confirm these results, whole lung histology slides were stained with trichrome blue to visualize collagen deposition after bleomycin administration. Again, there was an abundance of trichrome staining in the WT$\rightarrow$WT and WT$\rightarrow$IRAK-M$^{-/-}$ groups at day 21 after bleomycin and considerably less in both the IRAK-M$^{-/-}$$\rightarrow$IRAK-M$^{-/-}$ and IRAK-M$^{-/-}$$\rightarrow$WT groups (Fig. 4B). These data indicate that the presence of IRAK-M in the bone marrow compartment, rather than the structural cells, mediated the enhanced susceptibility to bleomycin-induced pulmonary fibrosis.

**Increased production of profibrotic molecules in WT but not IRAK-M$^{-/-}$ mice after bleomycin challenge**

It has previously been shown that cellular mediators play an important role in regulating the development and progression of lung fibrosis. We next assessed the expression of both pro- and anti-fibrotic molecules in whole lung or BALF samples obtained from WT and IRAK-M$^{-/-}$ mice after in vivo bleomycin challenge. Measurement of TGF-β using a luciferase activity assay revealed no differences in the amount of active TGF-β in BALF or whole lung samples isolated from WT and IRAK-M$^{-/-}$ mice (data not shown). Moreover, TNF-α, IL-1β, and GM-CSF were also measured, and there was no quantitative difference detected between groups (data not shown). Conversely, there was a significant increase in the production of IL-13 in BALF from WT, but not IRAK-M$^{-/-}$ mice after bleomycin challenge (Fig. 5A). To determine whether lung macrophages were producing IL-13 in our model, these cells were isolated by collagenase digestion from whole lungs of WT and IRAK-M$^{-/-}$ mice after bleomycin challenge. There was an increase in the production of IL-13 in WT, but not IRAK-M$^{-/-}$ macrophages after induction of fibrosis (Fig. 5B).

**FIGURE 4.** IRAK-M expression in the bone marrow compartment enhances susceptibility to collagen deposition after bleomycin-induced pulmonary fibrosis. Bone marrow chimeras were generated by transplanting WT or IRAK-M$^{-/-}$ BM into lethally irradiated WT or IRAK-M$^{-/-}$ mice. After complete BM reconstitution, mice were given an i.t. challenge with bleomycin, and whole lungs were collected at day 0, 14, or 21 after challenge. The amount of collagen was measured by hydroxyproline assay (A) as well as trichrome staining (B) of histological sections. Graphs represent mean ± SEM with n = 4 samples/group from two separate experiments. Original magnification ×200. *p < 0.05 compared with day 0 WT$\rightarrow$WT BMT mice.
These data indicate that IRAK-M is required for the production of IL-13 after bleomycin challenge.

**IRAK-M skews macrophages toward an alternatively activated phenotype after bleomycin administration**

Macrophage activation phenotypes are shaped by microenvironment factors, including cytokines. To determine whether increased production of IL-13 from macrophages corresponded to an alternatively activated macrophage phenotype, we assessed macrophage activation after bleomycin challenge. Lung macrophages were isolated from WT and IRAK-M−/− mice, and the expression of classically (inducible NO synthase [iNOS]) and alternatively activated (arginase, Fizz1) macrophage markers was determined by real-time RT-PCR. There was a significant increase in the expression of arginase (9-fold increase at day 7 and 18-fold increase at day 14; Fig. 6A) and Fizz1 (8.2-fold increase at day 7; Fig. 6B) in WT, but not IRAK-M−/−, lung macrophages. Conversely, there was a significant increase in the expression of iNOS in IRAK-M−/−, but not WT, lung macrophages at day 7 (16.6-fold increase at day 7; Fig. 6C). Activation of STAT6, an important transcription factor driving alternative activation in macrophages, was measured after bleomycin. There was a significant increase in the p-STAT6 relative to total STAT6 (and normalized to β-actin expression) in the WT, but not the IRAK-M−/−, lung macrophages after bleomycin challenge (Fig. 6D). No difference was noted in the expression of p-STAT1 compared with total STAT1 in these lung macrophage samples (data not shown). These data show that the absence of IRAK-M prevents the skewing of lung macrophages toward an alternatively activated phenotype.

To further define the role of IRAK-M in alternative macrophage activation, lung macrophages and isolated bone marrow–derived macrophages (BMDM) from WT and IRAK-M−/− mice were treated with either rmIL-13 alone or rmIL-4 and rmIL-13 in combination for 24 h, and then expression of macrophage activation markers was measured. A significant increase in the expression of Fizz1 (11.3-fold increase after IL-13 and 34.5-fold increase after IL-4/IL-13; Fig. 7A) and arginase (14-fold increase after IL-13 and 13.6-fold increase after IL-4/IL-13; Fig. 7B) was detected in WT, but not IRAK-M−/− lung macrophages after incubation with either rmIL-13 alone or in combination with rmIL-4. This effect was not specific for lung macrophages, as BMDM from WT mice showed substantially greater expression of Fizz1 and arginase after treatment with IL-13, as compared with IRAK-M−/− BMDM (Fig. 7C). BMDM were highly responsive to IL-13 treatment with 2630-fold increase in Fizz1 expression (Fig. 7C) and a 275-fold increase in arginase expression (Fig. 7D) compared with untreated controls. Additionally, incubation with exogenous rmIL-13 induced a significant increase in IRAK-M mRNA expression (2.2-fold increase; Fig. 7D) and protein expression (Fig. 7E) in lung macrophages. These data indicate that IRAK-M promotes IL-4/IL-13–mediated skewing of lung macrophages toward an alternatively activated phenotype, and IL-13 produced by alternatively activated macrophages can reciprocally induce IRAK-M.

**Alternatively activated macrophages increase expression of profibrotic molecules from fibroblasts**

To assess the effect of macrophages on fibroblast effector functions, an in vitro coculture system was established. First, fibroblasts grown from the lungs of unchallenged WT mice were serum starved for 24 h. Lung macrophages isolated (as described in Materials and Methods) from both unchallenged and day 14 after bleomycin-challenged WT and IRAK-M−/− mice were incubated either alone or in coculture with primary resting lung fibroblasts obtained from WT mice. There was no difference in the expression of alpha smooth muscle actin (α-SMA) (Fig. 8A) or collagen III (Fig. 8B) in day 0 macrophages cultured alone or with fibroblasts. However, when macrophages isolated from day 14 bleomycin-challenged WT and IRAK-M−/− mice were cocultured with lung fibroblasts, there was a significant increase in the expression of profibrotic molecules (Fig. 8A, 8B). Importantly, lung macrophages from WT mice cocultured with fibroblasts elicited a significantly higher expression (1.9-fold increase in α-SMA and 18.1-fold increase in collagen III) of profibrotic markers compared with IRAK-M−/− lung macrophages cocultured with fibroblasts. Furthermore, we measured the amount of active TGF-β in the supernatants from coculture experiments and found no differences in the production of TGF-β between cocultures utilizing IRAK-M−/− macrophages as compared with WT macrophages (data not shown).

To confirm that IRAK-M does not directly regulate lung fibroblast effector responses, primary fibroblasts were isolated from the lungs of WT or IRAK-M−/− mice and treated with TGF-β in vitro, and the expression of collagen I and IRAK-M was measured by Western blotting. TGF-β induced a significant increase in the amount of collagen I in fibroblasts from both WT and IRAK-M−/− mice; however, there was no IRAK-M expression in unstimulated or stimulated cells (Supplemental Fig. 2). To determine whether cell-to-cell contact was important in regulating collagen production by fibroblasts, macrophage-conditioned media was collected from either unchallenged or day 14 bleomycin-challenged WT or IRAK-M−/− lung macrophages and cultured
with resting fibroblasts. Incubation of supernatants from WT or IRAK-M<sup>−/−</sup> untreated macrophages did not cause changes in collagen expression; however, supernatants isolated from day 14 bleomycin-challenged WT, but not IRAK-M<sup>−/−</sup>, macrophages resulted in a significant increase in collagen III expression (Fig. 8C).

Previous work has shown that lung macrophages produced IL-13 during experimental fibrosis (4). To further elucidate the mechanism by which macrophages regulate fibroblast effector functions and expression of profibrotic mediators, IL-13–neutralizing or IgG control Abs were added to the macrophage–fibroblast cocultures. Again, there was no induction collagen expression in lung fibroblasts cocultured with resting WT or IRAK-M<sup>−/−</sup> macrophages (Fig. 8D). Similarly, after bleomycin challenge, there was a significant increase in the expression of collagen III from WT, but not IRAK-M<sup>−/−</sup>, macrophage–fibroblast cocultures. Interestingly, neutralization of IL-13 significantly reduced the expression of collagen III in the day 14 WT macrophage coculture system, whereas there was no induction by the day 14 IRAK-M<sup>−/−</sup> macrophage–fibroblast cocultures (Fig. 8D). These data suggest that IL-13 produced by WT, but not IRAK-M<sup>−/−</sup>, macrophages after bleomycin challenge stimulated collagen expression from fibroblasts.

**Increased IRAK-M and arginase expression in whole blood cells isolated from IPF patients**

To determine whether IRAK-M and the alternative activation marker arginase were differentially expressed in leukocytes isolated from the whole blood of patients with pulmonary fibrosis, gene expression was measured using a Gene Expression Omnibus database from a previously published peripheral blood transcriptome from 30 normal healthy patients and 93 IPF patients (31). In this study, RNA was isolated from peripheral blood cells from normal healthy controls and IPF patients. The IPF patients had a consensus diagnosis of probable or definite IPF based on American Thoracic Society/European Respiratory Society/Japanese Respiratory Society/Latin American Thoracic Association criteria and two pulmonary function measurements (lung diffusion capacity ≤ 65% and/or forced vital capacity ≤ 75%). These data revealed significantly higher expression of IRAK-M in peripheral blood cells from IPF patients as compared with healthy controls (Fig. 9A). Conflicting reports regarding the expression of arginase and other alternatively activated macrophage markers in IPF patients have been published (10, 11). To determine whether differences in macrophage activation markers were present in our cohort, we measured gene expression of arginase in peripheral blood cells from IPF or normal healthy control subjects. There was significantly elevated arginase expression from peripheral blood isolated from IPF patients compared with healthy controls (Fig. 9B). Additionally, IRAK-M mRNA levels positively correlated with arginase expression in peripheral blood from IPF patients (Fig. 9C). These data suggest a possible causal link between IRAK-M expression and alternative activation markers in circulating leukocytes of IPF patients.

**Discussion**

IRAK-M is an important regulator of both infectious and noninfectious lung injury; however, the contribution of this molecule to the pathogenesis of pulmonary fibrosis has yet to be determined. Using a murine model of experimental pulmonary fibrosis, we demonstrated that the presence of IRAK-M, specifically in the bone marrow compartment, promotes fibrogenesis, resulting in increased lung injury and collagen deposition. The expression of IRAK-M in macrophages skews these cells toward a profibrotic alternatively
activated phenotype. Elevated collagen production was observed when alternatively activated WT macrophages or cell-free supernatants from bleomycin-challenged mice were cocultured with fibroblasts. Additionally, inhibition of IL-13 in WT macrophages–fibroblasts resulted in decreased collagen expression in our coculture system. These data shed light on the growing evidence that negative regulators of TLR signaling pathways play an important role not only in controlling inflammatory responses, but also in regulating lung repair and remodeling mechanisms.

Our data reveal a critical role for IRAK-M in promoting bleomycin-induced pulmonary fibrosis. IRAK-M−/− mice had increased early neutrophilic influx, but reduced later accumulation of both neutrophils and macrophages. Diminished cellular influx into the lungs of IRAK-M−/− mice later after bleomycin challenge correlated with reduced lung injury. The enhanced early influx of neutrophils in IRAK-M−/− mice was anticipated and has been observed in other inflammatory model systems (25); however, reduced inflammation in these mice at later time points (7 d) is somewhat surprising. The skewing toward alternative macrophage activation and the production of IL-13 in bleomycin-challenged WT mice may induce the production of chemotactic factors that facilitate later pulmonary inflammation. Previous work has shown IL-13 can induce CCL2 expression from the endothelium to promote leukocyte recruitment (32). Additionally, IL-13 is a known inducer of peristin, which can mediate cellular recruitment (33). However, we did not observe significant differences in the expression of CCL2 or peristin in bleomycin-challenged IRAK-M−/− mice as compared with WT animals (data not shown). Protection against lung injury in bleomycin-challenged IRAK-M−/− mice also suggested that IRAK-M could be promoting epithelial cell death. Our laboratory has previously shown that IRAK-M impairs the production of antioxidant molecules from AECs after hyperoxic lung injury (26). Although we observed a modest, but significant increase in IRAK-M mRNA expression in AECs after bleomycin challenge (Supplemental Fig. 1A), we did not detect differences in expression of glutathione peroxidase-2 and heme-oxygenase-1 in AECs after injury (data not shown). These data, along with our chimera results, suggest that expression of IRAK-M in AECs or other structural cells does not meaningfully contribute to the fibroproliferative response after bleomycin administration.

Previous work has suggested a possible role for IRAK-M in remodeling and repair events processes. Specifically, genetic deletion of this negative TLR regulator protected mice against acute kidney injury and myocardial infarction (21, 34). In these models, IRAK-M−/− mice have increased expression of proinflammatory mediators and enhanced renal fibrosis after kidney injury (34) and enhanced myocardial inflammation and adverse remodeling after infarction (21). Our findings are somewhat at odds with these reports. An important distinction is that in these aforementioned injury models, classically activated proinflammatory macrophages promote disease in both acute kidney injury and myocardial infarctions models (35, 36), whereas in the bleomycin model, classically activated macrophages prevent the development of pulmonary fibrosis (9, 11, 37, 38). These contrasting results highlight how the influence of macrophages on reparative pro-
Although differences in the role of IRAK-M in regulating injury exist, a consistent theme across disease states is that IRAK-M promotes alternative activation of macrophages. Our data demonstrate that after bleomycin challenge or incubation with rmIL-13, IRAK-M−/− lung macrophages maintain a classically activated phenotype (as indicated by decreased Fizz1 and arginase expression, increased iNOS expression, and reduced STAT-6 phosphorylation) compared with WT macrophages (Figs. 6, 7). This is not a lung-specific phenomenon, as WT BMDM also displayed increased expression of Fizz1 and arginase when treated with rmIL-13 in vitro, and observed no difference in mRNA expression of known markers or production of proinflammatory mediators after stimulation rather than with functional assays. It has yet to be determined whether IRAK-M alters the ability of macrophages to differentially metabolize arginine into either NO via classical activation or urea through alternative activation.

Mechanisms leading to upregulation of IRAK-M in our model have not been completely defined. Previous work has shown that a variety of endogenous molecules, including hyaluronan, collagen, fibronectin, and other matrix components, can bind to TLRs and induce intracellular signaling pathways (13). We have yet to identify the TLR ligand in our bleomycin injury model. It has been shown that IRAK-M can be induced in response to several pathogen-associated molecular patterns, most notably LPS. There are a number of factors present in the lung microenvironment regulating injury and reparative responses, which may do so by regulating the expression of IRAK-M (22, 25, 26). In particular, we found that the profibrotic mediator IL-13 induced IRAK-M expression in lung macrophages, an effect similar to that previously shown in airway epithelial cells (22). The endogenous lung molecule, surfactant protein A, can also stimulate IRAK-M expression in human macrophages (39). A recent report demonstrated that collagen monomers upregulate alternatively activated markers in human alveolar macrophages, which suggests an amplification loop between macrophage activation and extracellular matrix molecules (40). The contribution of IRAK-M to collagen-induced alternative activation was not explored. Alternatively activated macrophages can also produce collagen, which could lead to increased fibrosis (41). To this end, we measured collagen expression in WT and IRAK-M−/− macrophages treated with rmIL-13 in vitro, and observed no difference in mRNA expression of collagen in any of these groups (data not shown).
Our findings indicate that IRAK-M−/− mice produce less IL-13 after bleomycin challenge (Fig. 5A). Previous work has shown the importance of IL-13 in the development and maintenance of pulmonary fibrosis (5, 6). Several clinical trials are currently underway to determine whether inhibition of IL-13 can ameliorate or halt the progression of pulmonary fibrosis (1). Using a unique humanized SCID pulmonary fibrosis model, inhibition of IL-13 blocks lung remodeling after the establishment of fibrosis as well as promoting lung repair and restoring epithelial integrity (42). We also observed reduced expression of IL-13 from IRAK-M–deficient macrophages harvested from bleomycin-challenged mice ex vivo. The precise mechanism accounting for reduced IL-13 production in IRAK-M–deficient cells is unclear, but we found impaired activation of STAT-6 in IRAK-M−/− macrophages, a transcription factor required for alternative macrophage activation and IL-13 production. Recently it has been reported that IL-13–stimulated platelet-derived growth factor induced collagen I production in airway fibroblast in a JAK/STAT6-dependent mechanism (43). Whereas lung macrophages are thought to be a major cellular source of IL-13 during fibrogenesis, there are other cell types that may also contribute to IL-13 production. Bleomycin-induced pulmonary fibrosis has been shown to be a T cell–independent model; therefore, production of IL-13 by this cell population is likely not responsible for regulating fibrogenesis in our model (44).

However, a novel population of cells, type 2 innate lymphoid cells (ILC2), has recently been shown to drive collagen deposition after bleomycin challenge, and increased numbers of ILC2s were found in the lungs of pulmonary fibrosis patients (45). More importantly, it has been shown that ILC2s can promote alternative activation of macrophages via the production of IL-13 (46). Whether these cells express IRAK-M and whether IRAK-M regulates IL-13 production in these cells is an ongoing area of investigation.

In summary, our study illuminates a novel role of IRAK-M in regulating macrophage activation phenotypes and fibrogenesis during the evolution of bleomycin-induced pulmonary fibrosis. This work motivates future studies to explore the pathogenic role of IRAK-M in IPF and as a potential biomarker in pulmonary fibrosis patients.

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Disclosures
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References


Supplemental Figure 1: Increased IRAK-M in AECs but not fibroblasts after bleomycin challenge

WT mice were given an i.t. dose of bleomycin and AECs and lung fibroblasts were isolated. The amount of IRAK-M expression was measured by realtime RT-PCR in AECs (A) and fibroblasts (B). Graphs represent mean ± SEM with n=4 samples/group from 2 separate experiments.
Supplemental Figure 2: No expression of IRAK-M in lung fibroblasts

Whole lung fibroblasts were grown from WT and IRAK-M⁻/⁻ mice. Cells were either treated with TGF-β (10ng/ml) or media alone for 24 hrs. The amount of collagen I, IRAK-M and GAPDH (housekeeping gene) was measured by western blot analysis.