Susceptibility of Signal Transducer and Activator of Transcription-1-Deficient Mice to Pulmonary Fibrogenesis

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The signal transducer and activator of transcription (Stat)-1 mediates growth arrest and apoptosis. We postulated that lung fibrosis characterized by excessive proliferation of lung fibroblasts would be enhanced in Stat1-deficient (Stat1^{-/-}) mice. Two weeks after bleomycin aspiration (3 U/kg), Stat1^{-/-} mice exhibited a more severe fibroproliferative response and significantly elevated total lung collagen compared to wild-type mice. Growth factors [epidermal growth factor (EGF) or platelet-derived growth factor (PDGF)] enhanced [³H]thymidine uptake in lung fibroblasts isolated from Stat1^{-/-} mice compared to wild-type mice. Interferon (IFN)- γ , which signals growth arrest via Stat1, inhibited EGF- or PDGF-stimulated mitogenesis in wild-type fibroblasts but enhanced [³H]thymidine uptake in Stat1^{-/-} fibroblasts. Moreover, IFN- γ treatment in the absence of growth factors induced a concentration-dependent increase in [³H]thymidine uptake in Stat1^{-/-} but not wild-type fibroblasts. Mitogen-activated protein kinase (ERK-1/2) phosphorylation in response to PDGF or EGF did not differ among Stat1^{-/-} and wild-type fibroblasts. However, Stat3 phosphorylation induced by PDGF, EGF, or IFN- γ increased twofold in Stat1^{-/-} fibroblasts compared to wild-type fibroblasts. Our findings indicate that Stat1^{-/-} mice are more susceptible to bleomycin-induced lung fibrosis than wild-type mice due to 1) enhanced fibroblast proliferation in response to growth factors (EGF and PDGF), 2) stimulation of fibroblast growth by a Stat1-independent

IFN- γ signaling pathway, and 3) increased activation of Stat3. (Am J Pathol 2005, 167:1221–1229)

Pulmonary fibrosis is characterized by the excessive deposition of collagen in alveolar regions resulting in thickened alveolar septae and subsequent impairment of gas exchange. Lung fibroblasts are the central cell type that secrete collagen and other extracellular matrix proteins to define the fibrotic lesion.¹ Proliferation of the fibroblasts within the lung is therefore a key feature in the development of fibrosis. Fibroblast proliferation is driven by several polypeptide growth factors that are up-regulated after lung injury, including platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptor ligands.^{2,3} The action of these growth factors in promoting cell division is mediated by the phosphorylation of their specific receptor tyrosine kinases and the subsequent downstream activation of mitogen-activated protein (MAP) kinase cascades and transcription factors.⁴

The signal transducer and activator of transcription (Stat) proteins are pivotal regulators of cell survival and proliferation or growth arrest and apoptosis.⁵ Of the seven mammalian Stats that have been identified, Stat1, Stat3, and Stat5 are activated by polypeptide growth factors including PDGF and EGF. Activated Stat1 is a signal for growth arrest and apoptosis, whereas Stat3 mediates cell growth and survival.⁶ Although the overall response of fibroblasts to PDGF and EGF is promitogenic, these growth factors also generate a growth inhibitory signal via Stat1.

Interferon- γ (IFN- γ) is a potent activator of Stat1 yet does not activate other members of the Stat family.⁵ IFN- γ

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signals growth arrest through a Stat1-dependent mechanism and inhibits PDGF- or EGF-stimulated fibroblast growth.^{7,8} Therefore, IFN- γ overwhelms the promitogenic activity of growth factors that are mediated via Stat3. Recently, a great deal of attention focused on a preliminary clinical trial that implicated IFN- γ as a beneficial therapy for idiopathic pulmonary fibrosis.⁹ The rationale for these investigations hinges on the ability of IFN- γ to arrest myofibroblast growth through the Stat1 signaling pathway. To date such studies have yielded mixed results and it remains controversial whether or not IFN- γ is an effective therapy for fibrotic diseases.

We previously reported that metal-induced oxidative stress strongly activates Stat1 in rat lung fibroblasts *in vitro*.¹⁰ However, the susceptibility of Stat1^{-/-} mice to pulmonary fibrosis has not yet been investigated. Stat1^{-/-} mice show no overt developmental abnormalities, but display a complete lack of responsiveness to either IFN- γ or IFN- α and are susceptible to infection by microbial pathogens.^{11,12} In the present study, we show that the fibrotic response after bleomycin-induced lung injury was enhanced in Stat1^{-/-} mice compared to wild-type mice. Additionally, we report that the growth response to EGF and PDGF in fibroblasts isolated from the lungs of Stat1^{-/-} mice was enhanced. Although IFN- γ inhibited EGF- and PDGF-induced growth in wild-type lung fibroblasts, mitogenesis stimulated by these growth factors was significantly enhanced by IFN- γ in lung fibroblasts from Stat1^{-/-} mice. Moreover, IFN- γ stimulated the growth of Stat1^{-/-} lung fibroblasts in the absence of other growth factors, yet had no mitogenic activity for lung fibroblasts from wild-type mice. We also found that Stat1^{-/-} lung fibroblasts exhibit enhanced Stat3 phosphorylation compared to wild-type lung fibroblasts after stimulation with PDGF, EGF, or IFN- γ . Collectively our study provides new evidence that Stat1 is protective against the development of pulmonary fibrosis.

Materials and Methods

Animals

Male 129S6/SvEv (wild-type) and Stat1^{-/-} mice (5 to 6 weeks old) were purchased from Taconic Farms, Germantown, NY. Animals were provided with food and water *ad libitum*. Experimental protocols for mice were conducted in accordance with the standards established by the US Animal Welfare Act, set forth in National Institutes of Health guidelines. Mice were given bleomycin sulfate (3 U/kg) in 50 μ l of phosphate-buffered saline (PBS) or PBS via oropharyngeal aspiration. Mice were euthanized 14 days after bleomycin or PBS challenge. Lungs were lavaged with 1 ml of cold PBS-calcium magnesium-free for collection of bronchoalveolar lavage cells. Lavaged lungs were used for determination of lung hydroxyproline content. An equal number of lavaged lungs and unlabeled lungs were fixed in 10% formalin and used for histological evaluation.

Histology and Pathology Scoring

Formalin-fixed lungs were embedded in paraffin and cut in 5- μ m sections. Sections were stained with Masson's trichrome. Sections were scored blindly, and the volume fraction of each component was calculated based on the principle that volume fraction of each component is theoretically equal to point fraction of each component.^{13,14} Five $\times$ 10 pictures were taken randomly from the left lung. A point grid file (35 points) was overlaid on each picture, and every component that was hit by a point grid was identified as alveolar space, bronchi and bronchioles, alveolar walls including infiltrating cells, blood and lymphatic vessels, or the outer space of the lung for a total 175 points to calculate point fraction of each component. The number of points for each component from five pictures was nested for each animal, and the point fraction of each animal was calculated by dividing the number of points hitting each component by the number of points hitting any components of the lung (except the outer space). Then, as an index of interstitial fibrosis, the ratio of volume fraction of alveolar walls to volume fraction of alveolar space (W/A ratio) was calculated for each animal.

Hydroxyproline Assay

The procedure for quantitation of lung hydroxyproline has been described elsewhere.¹⁵ Whole lung tissue was washed in PBS and hydrolyzed for 18 hours in 6 N HCl at 110°C (~40 ml/6 g of tissue). One drop of 1% phenolphthalein in ethanol was added to each sample and the pH adjusted to 6.0 with NaOH titration. Two ml from each sample was centrifuged 5 minutes at 1500 rpm and the pellet oxidized with 1 ml of 0.6 mol/L chloramine-T for 30 minutes. One ml of 7.5% *p*-dimethylaminobenzaldehyde was added to each sample and samples were incubated at 65°C for 15 minutes. The absorbance was measured at 560 nm on a spectrophotometer. Lung hydroxyproline was quantitated against a standard curve of purified hydroxyproline (Sigma, St. Louis, MO) and values were corrected for total lung wet weight.

Cell Culture

Lung fibroblasts were isolated from Stat1^{-/-} and wild-type mice according to a previously established protocol.¹⁶ Second passage cells (10⁶) were seeded in 75-cm² flasks and grown to 75% confluence in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum plus antibiotics.

[³H]Thymidine Assay

Cells were trypsin-liberated and each flask was split into two 24-well cell culture plates. Cells were grown overnight in Dulbecco's modified Eagle's medium/5% fetal bovine serum, then quiesced in Dulbecco's modified Eagle's medium with 0.5% fetal bovine serum for 48 hours. Cells were treated with growth factors (EGF or PDGF-BB)

or IFN- γ for 24 hours followed by incubation with [³H]thymidine (2 μ Ci/ml) for 3 hours. Cells were washed, placed on ice, and incubated with 5% trichloroacetic acid for 10 minutes. After additional washing with ice-cold water, cells were solubilized with 0.2 N NaOH containing 0.1% sodium dodecyl sulfate. One hundred ml per sample was added to 1 ml of scintillation fluid and radioactivity was measured on a liquid scintillation counter.

Western Blot Analysis

Equal amounts of protein (20 μ g) from each sample were separated on 10 to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels for Stat proteins or ERK MAP kinase. Proteins were then transferred to a nitrocellulose membrane. The membrane was blocked for 2 hours at room temperature with 5% nonfat milk in TBS-Tween buffer (20 mmol/L Tris, 500 mmol/L NaCl, 0.01% Tween 20). The blot was then incubated with a 1:1000 dilution of the anti-phospho-STAT-3 (New England Biolabs, Beverly, MA), anti-Stat3 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-ERK or anti-ERK (Cell Signaling Technologies, Inc., Beverly, MA) at 4°C overnight, followed by incubation for 1 to 2 hours with the secondary horseradish peroxidase-conjugated anti-rabbit IgG (1:2000 dilution). The immunoblot was visualized through enhanced chemiluminescence or chemifluorescence.

Real-Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction

Total RNA from human lung fibroblasts was isolated using the RNeasy Miniprep kit (Qiagen, Valencia, CA). Two μ g of total RNA was reverse-transcribed at 48°C for 30 minutes using Multiscribe reverse transcriptase (Applied Biosystems, Foster City, CA) in 1 $\times$ reverse transcriptase buffer, 5.5 mmol/L MgCl₂, 0.5 μ mol/L of each dNTP, 2.5 μ mol/L of random hexamers, and 0.4 U/ μ l RNase inhibitor in a volume of 100 μ l. Forty ng of the reverse transcriptase product was amplified using TaqMan gene expression assays specific for PDGF-A and β -actin on the 7700 Prism sequence detection system (Applied Biosystems, Foster City, CA). The primer sequence for mouse PDGF-A was ATAGACTCCGTAGGGGCTGAGGATG and for mouse β -actin was TGTACTGAGCTGCGTTTACACCC. The polymerase chain reaction conditions and data analysis were performed according to the manufacturer's protocol described in user bulletin no.2, Applied Biosystems Prism 7700 Sequence Detection System. All samples were run in triplicate. Gene expression was measured by the quantitation of cDNA converted from mRNA corresponding to PDGF-A relative to the untreated 0-minute control groups and normalized to β -actin. Relative quantitation values were expressed as fold-change.

Statistics

Data are expressed as mean $\pm$ SEM or SD as indicated. A two-way analysis of variance was used to analyze

differences between strains and treatment groups with post hoc comparisons by the Holm-Sidak method (mitogenesis and hydroxyproline data) or paired Student's *t*-test (ERK and Stat3 densitometry data). A value of *P* < 0.05 was considered significant.

Results

Lung Fibrosis Was Increased in Stat1^{-/-} Mice Compared to Wild-Type Mice after Bleomycin-Induced Lung Injury

The lungs from wild-type and Stat1^{-/-} mice were histologically normal after PBS aspiration (Figure 1). Trichrome staining of histological sections demonstrated that bleomycin caused lung fibrosis characterized by collagen deposition after 2 weeks in both wild-type and Stat1^{-/-} mice (Figure 1). However, honeycomb lesions with more abundant air spaces were found in the lungs of wild-type mice, whereas the lungs of Stat1^{-/-} mice contained dense, collagen-laden fibrotic lesions. Histological scoring and hydroxyproline assays confirmed that bleomycin induced a more severe fibrotic response in Stat1^{-/-} mice than in wild-type mice. A greater ratio of tissue volume/airspace volume was found in the lungs of Stat1^{-/-} mice compared to wild-type mice (Table 1). Furthermore, bleomycin increased total lung collagen in wild-type and Stat1^{-/-} mouse lungs, yet the lungs of Stat1^{-/-} mice contained significantly greater levels of collagen as assessed by hydroxyproline assay (*P* < 0.01) compared to wild type (Figure 2). Bleomycin also caused significant inflammation in both strains of mice. The total number of cells recovered in bronchoalveolar lavage fluid from wild-type and Stat1^{-/-} mice was increased in bleomycin-treated mice compared to PBS-treated animals, although this increase was significantly greater in Stat1^{-/-} mice (Table 2). Additionally, numbers of bronchoalveolar lavage macrophages, epithelial cells, neutrophils, eosinophils, and lymphocytes were increased in Stat1^{-/-} mice.

Proliferative Responses to EGF and PDGF in Stat1^{-/-} Fibroblasts Were Enhanced by IFN- γ

A significantly increased, dose-dependent mitogenic response to EGF and PDGF-BB was found in Stat1^{-/-} mouse lung fibroblasts compared to those from wild-type mice (Figures 3 and 4). IFN- γ has been reported to inhibit the mitogenic response to polypeptide growth factors *in vitro*, including EGF and PDGF.⁸ Co-incubation of wild-type mouse lung fibroblasts with IFN- γ completely inhibited EGF- and PDGF-BB-induced mitogenesis, whereas IFN- γ significantly enhanced the mitogenic responses of EGF and PDGF-BB in Stat1^{-/-} mouse lung fibroblasts (Figures 3 and 4). These data indicated that IFN- γ enhances the promitogenic effect of growth factors on fibroblasts in a Stat1-independent manner.

Figure 1. Masson's trichrome staining of representative histological sections from the left lungs of wild-type (WT) and *Stat1*^{-/-} mice 2 weeks after treatment with PBS or 3 U/kg of bleomycin sulfate. Blue staining indicates mature collagen. Bleomycin-treated *Stat1*^{-/-} mice typically had more compact lung fibrotic lesions with more intense trichrome staining as compared to the honeycomb fibrotic lung lesions commonly observed in WT mice. Original magnifications: $\times 10$; $\times 40$ (insets).

*IFN- γ Stimulates the Proliferation of *Stat1*^{-/-}, but Not Wild-Type, Mouse Lung Fibroblasts in Vitro*

In addition to enhancing the mitogenic activity of EGF and PDGF-BB, we also observed that IFN- γ exerted a mitogenic effect on *Stat1*^{-/-} lung fibroblasts in a concentration-dependent manner in the absence of other growth factors (Figure 5). In contrast, no mitogenic response to IFN- γ was found in lung fibroblasts from wild-type mice.

These data indicated that IFN- γ stimulates mitogenesis through a *Stat1*-independent mechanism.

Potential *Stat1*-independent mechanisms include release of autocrine growth factors and/or enhanced activation of signaling intermediates. We investigated the role of PDGF-AA as a candidate autocrine growth factor. A neutralizing antibody to PDGF-AA had no effect on IFN- γ -induced proliferation of *Stat1*^{-/-} fibroblasts (data not shown). Moreover, quantitative reverse transcriptase-polymerase chain reaction time course ex-

Table 1. Histological Scoring of Lung Sections

Treatment	Strain	Volume fraction (%) [*]				
		V _W	V _A	V _B	V _V	W/A [†]
PBS	WT	26.4 $\pm$ 0.8	53.5 $\pm$ 3.4	14.5 $\pm$ 3.0	5.7 $\pm$ 0.9	0.500 $\pm$ 0.038
Bleomycin	WT	41.8 $\pm$ 7.4	39.3 $\pm$ 5.2	12.3 $\pm$ 2.2	6.6 $\pm$ 2.0	1.241 $\pm$ 0.448
PBS	<i>Stat1</i> ^{-/-}	26.7 $\pm$ 1.9	55.9 $\pm$ 2.9	11.5 $\pm$ 2.7	5.9 $\pm$ 1.1	0.479 $\pm$ 0.038
Bleomycin	<i>Stat1</i> ^{-/-}	46.0 $\pm$ 3.2	34.9 $\pm$ 1.7	10.3 $\pm$ 1.0	8.7 $\pm$ 1.4	1.338 $\pm$ 0.155

Scoring of lung sections was performed as described in Materials and Methods. Data represent the mean and SD of four mice.

^{*}Volume fraction of alveolar walls included infiltrating cells (V_W), alveolar space (V_A), bronchi and bronchioles (V_B), and blood and lymphatic vessels (V_V).

[†]Ratio of V_W to V_A.

Figure 2. Hydroxyproline content in lungs from wild-type (filled bars) and Stat1^{-/-} mice (open bars) 2 weeks after treatment with PBS or 3 U/kg bleomycin sulfate. Data are the mean $\pm$ SEM of six mice in the PBS groups and seven mice in the bleomycin groups. ** $P < 0.01$ versus PBS control within strain. $^{\S}P < 0.01$ versus WT for bleomycin treatment.

periments showed that IFN- γ increased PDGF-AA mRNA expression in wild-type mouse lung fibroblasts ~ 1.5 -fold within 30 minutes with a gradual increase to 2.5-fold by 24 hours after treatment (data not shown). In contrast, IFN- γ did not increase PDGF-AA mRNA expression in Stat1^{-/-} mouse lung fibroblasts within the first 12 hours of treatment and only stimulated a 1.5-fold increase by 24 hours (data not shown). Taken together, these data suggest that PDGF-AA did not mediate IFN- γ -induced proliferation of Stat1^{-/-} lung fibroblasts. This does not rule out the possibility that another mitogen(s) could be mediating the growth response to IFN- γ in Stat1^{-/-} fibroblasts.

To address the possibility that Stat1^{-/-} mouse lung fibroblasts have altered intracellular signaling that confers enhanced growth responses, we investigated two major intracellular signaling intermediates involved in cell survival and growth responses, ERK and Stat3. IFN- γ did not stimulate the phosphorylation of ERK in either wild-type or Stat1^{-/-} lung fibroblasts, whereas EGF and

Figure 3. DNA synthesis induced by EGF in the absence or presence of IFN- γ in mouse lung myofibroblasts isolated from wild-type (WT) or Stat1^{-/-} mice. IFN- γ (100 U/ml) inhibited the EGF-induced mitogenic response in WT fibroblasts. In contrast, IFN- γ enhanced EGF-induced mitogenesis in Stat1^{-/-} lung fibroblasts. DNA synthesis was measured by [3 H]thymidine uptake as described in Materials and Methods. Data are the mean $\pm$ SEM of four separate experiments each performed in quadruplicate. * $P < 0.05$ versus media control (no growth factor or IFN- γ). + $P < 0.05$ versus GF only within strain. $n = 4$ cultures/group.

PDGF stimulated the phosphorylation of ERK (Figure 6). However, IFN- γ stimulated Stat3 phosphorylation and Stat1^{-/-} lung fibroblasts showed a twofold increase in phospho-Stat3 levels compared to wild-type cells in response to IFN- γ (Figure 7).

PDGF- and EGF-Induced Phosphorylation of Stat3 Was Enhanced in Stat1^{-/-} Lung Fibroblasts

To address the possibility that Stat1^{-/-} mouse lung fibroblasts have altered intracellular signaling that confers enhanced growth responses, we examined the activation state of ERK and Stat3, two major intracellular signaling intermediates involved in cell survival and growth responses. ERK-1/2 phosphorylation, in response to EGF or PDGF, was not different between Stat1^{-/-} lung fibroblasts and wild-type fibroblasts and IFN- γ did not further

Table 2. Cells Recovered in the Bronchoalveolar Lavage Fluid from Bleomycin- or PBS-Treated Wild-Type (WT) or Stat1^{-/-} Mice after 24 Hours

Strain	Treatment	Macrophages	Epithelial	Neutrophils	Eosinophils	Lymphocytes
WT	Control	4.67 $\pm$ 0.52	0.92 $\pm$ 0.12	0.03 $\pm$ 0.01	0.01 $\pm$ 0.005	0.006 $\pm$ 0.006
	Bleomycin	7.06 $\pm$ 1.43	0.35 $\pm$ 0.09*	0.16 $\pm$ 0.06	0.13 $\pm$ 0.07	0.31 $\pm$ 0.07*
Stat1 ^{-/-}	Control	3.73 $\pm$ 0.04	0.84 $\pm$ 0.11	0.71 $\pm$ 0.04	0.02 $\pm$ 0.005	0.04 $\pm$ 0.008
	Bleomycin	10.00 $\pm$ 1.60*	1.10 $\pm$ 0.05†	1.10 $\pm$ 0.33*†	0.21 $\pm$ 0.06*	0.56 $\pm$ 0.12*†

Data are expressed as the mean $\pm$ SEM of cell numbers $\times 10^4$ ($n = 6$ /group). The total numbers of BAL cells were not different between PBS-treated WT (5.66 $\pm$ 0.64) and Stat1^{-/-} mice (4.75 $\pm$ 0.14). A significant difference ($P < 0.05$) was observed between total BAL cells in bleomycin-treated WT (8.42 $\pm$ 1.37) and Stat1^{-/-} mice (13.15 $\pm$ 1.40).

* $P < 0.05$ versus PBS control within strain.

† $P < 0.05$ versus WT for same treatment.

Figure 4. DNA synthesis induced by PDGF-BB in the absence or presence of IFN- γ in mouse lung fibroblasts isolated from wild-type (WT) or Stat1^{-/-} mice. IFN- γ (100 U/ml) inhibited the PDGF-induced mitogenic response in WT fibroblasts. In contrast, IFN- γ enhanced PDGF-induced mitogenesis in Stat1^{-/-} lung fibroblasts. DNA synthesis was measured by [³H]thymidine uptake as described in Materials and Methods. Data are the mean $\pm$ SEM of four separate experiments each performed in quadruplicate. * P < 0.05 versus media control (no growth factor or IFN- γ). + P < 0.05 versus growth factor only within strain. n = 4 cultures/group.

enhance ERK phosphorylation (Figure 6). In contrast, EGF- or PDGF-stimulated phosphorylation of Stat3 was enhanced approximately twofold in Stat1^{-/-} lung fibroblasts compared to wild-type fibroblasts (Figure 7). Although IFN- γ alone stimulated Stat3 phosphorylation in lung fibroblasts from both strains, Stat1^{-/-} lung fibroblasts showed a twofold increase in phospho-Stat3 levels compared to wild-type cells (Figure 7). However, IFN- γ did not further increase EGF- or PDGF-stimulated Stat3 phosphorylation.

Figure 5. IFN- γ stimulates a dose-dependent mitogenic response in Stat1^{-/-} mouse lung fibroblasts, but not in lung fibroblasts from wild-type (WT) mice. DNA synthesis was measured by [³H]thymidine uptake as described in Materials and Methods. Data are the mean $\pm$ SEM of four separate experiments each performed in quadruplicate. * P < 0.05 versus growth factor only within strain. § P < 0.05 versus WT. n = 4 cultures/group.

Figure 6. Western blot analysis showing EGF- and PDGF-induced phosphorylation of ERK-1/2 in the absence or presence of IFN- γ (1000 U/ml) in fibroblasts isolated from the lungs of wild-type (WT) and Stat1^{-/-} mice. **A:** Phosphorylated ERK-1/2 (pERK-1/2) in lung fibroblasts from Stat1^{-/-} and WT mice after 10 minutes of treatment with EGF or PDGF-BB in the absence or presence of IFN- γ (1000 U/ml). IFN- γ did not stimulate ERK-1/2 phosphorylation in either WT or Stat1^{-/-} lung fibroblasts. **B:** Densitometric evaluation of ERK-1 activation. The relative level of ERK activation was determined by densitometric scanning of the phospho-ERK (p-ERK-1/2) bands and normalized to the total ERK protein signal (ERK-1/2). Data are the mean $\pm$ SEM of three independent experiments.

Discussion

In this study we have shown that Stat1^{-/-} mice are more susceptible than wild-type mice to bleomycin-induced pulmonary fibrosis. This is the first investigation that has established a definitive role for Stat1 during lung fibrogenesis. Significantly higher levels of collagen and more severe fibrotic lesions, as well as increases in inflammatory cells, were found in Stat1^{-/-} mice 2 weeks after bleomycin treatment compared to their wild-type counterparts. Using *in vitro* experiments with early passage mouse lung fibroblasts, we also provide a mechanistic basis for understanding the susceptibility of Stat1^{-/-} mice to bleomycin-induced lung fibrosis. These *in vitro* experiments show that 1) Stat1^{-/-} fibroblasts exhibit greater proliferative responses to EGF and PDGF compared to wild-type fibroblasts; 2) IFN- γ enhances EGF- and PDGF-stimulated mitogenesis in Stat1^{-/-} fibroblasts yet inhibits the effects of these growth factors in wild-type cells; 3) IFN- γ stimulates the mitogenesis of Stat1^{-/-} lung fibroblasts in the absence of other growth factors, yet

Figure 7. Western blot analysis showing EGF- and PDGF-induced phosphorylation of Stat3 in the absence or presence of IFN- γ in fibroblasts isolated from the lungs of wild-type (WT) and Stat1^{-/-} mice. **A:** Phosphorylated Stat3 (pStat3) in lung fibroblasts from Stat1^{-/-} and WT mice after 10 minutes of treatment with EGF or PDGF-BB in the absence or presence of IFN- γ (1000 U/ml). IFN- γ alone stimulated Stat3 phosphorylation in either WT or Stat1^{-/-} lung fibroblasts. **B:** Densitometric evaluation of Stat3 activation. The relative level of Stat3 activation was determined by densitometric scanning of the phospho-Stat3 (pStat3) bands and normalized to the total Stat3 protein signal. EGF-, PDGF-, or IFN- γ -stimulated Stat3 phosphorylation was twofold greater in lung fibroblasts from Stat1^{-/-} mice as compared to WT mice. Data are the mean $\pm$ SEM of three independent experiments. * P < 0.05 versus WT for treatment.

IFN- γ possesses no mitogenic effect on wild-type lung fibroblasts; and 4) Stat1^{-/-} lung fibroblasts exhibit enhanced phosphorylation of Stat3 compared to wild-type lung fibroblasts after stimulation with PDGF, EGF, or IFN- γ .

The enhanced proliferative response to EGF and PDGF in lung fibroblasts from Stat1^{-/-} mice could be a major factor in the enhanced susceptibility of these mice to bleomycin-induced lung fibrosis. PDGF is a potent mitogen and chemoattractant for cells of mesenchymal origin and numerous studies have implicated PDGF and its receptors in the pathogenesis of pulmonary fibrosis in humans and experimental animals.³ Ligands of the EGF receptor, including EGF, transforming growth factor- α , and HB-EGF, are also important mediators of fibrosis and contribute to the proliferative phase of fibrogenesis that involves mesenchymal cell replication.¹⁷⁻¹⁹ Importantly, receptor tyrosine kinase inhibitors selective for either the PDGFR or EGFR have been shown to reduce pulmonary

fibrosis in rats.¹⁵ Thus, increased sensitivity of Stat1^{-/-} lung fibroblasts to the mitogenic effects of EGF and PDGF receptor ligands could account for the more severe fibrotic lesions and accompanying increase in total lung collagen observed in Stat1^{-/-} mice after bleomycin-induced lung injury.

The underlying mechanism of enhanced EGF- and PDGF-induced mitogenesis in Stat1^{-/-} lung fibroblasts is not entirely clear. However, EGF and PDGF are known to activate anti-mitogenic signaling molecules (eg, p38 MAPK and Stat1) as well as promitogenic signaling molecules (eg, ERK and Stat3).²⁰⁻²² For example, metabolic inhibitors of p38 MAPK enhanced EGF- and PDGF-stimulated rat lung fibroblast mitogenesis *in vitro*, demonstrating p38 MAPK as a growth-suppressive signaling intermediate.¹⁶ Stat1 activated by EGF and PDGF also results in a growth-suppressive signal. Our data demonstrate that Stat1 deficiency disrupts this growth arrest pathway, resulting in enhanced cell proliferation in the presence of growth factors. In addition to the disruption of the growth arrest function of Stat1, we also showed that EGF- or PDGF-induced phosphorylation of Stat3 was enhanced in Stat1^{-/-} fibroblasts compared to wild-type fibroblasts. Stat3 is known to mediate prosurvival and promitogenic responses in fibroblasts. Our findings suggest that enhanced mitogenic responses to EGF and PDGF could be due to the lack of the growth arrest activity of Stat1 as well as enhanced activation of Stat3. Moreover, the constitutive increase in Stat3 activation suggests that Stat1 plays a role in regulating the phosphorylation or dephosphorylation status of Stat3.

In the present study, the effects of IFN- γ on lung fibroblast proliferation induced by either EGF or PDGF were markedly different among lung fibroblasts isolated from the lungs of Stat1^{-/-} mice and wild-type mice. IFN- γ has been reported to inhibit the PDGF-stimulated growth of normal rat lung fibroblasts.⁸ Similarly, we observed that IFN- γ inhibited EGF- or PDGF-stimulated mitogenesis in wild-type mouse lung fibroblasts. In contrast, fibroblasts isolated from the lungs of Stat1^{-/-} mice exhibited enhanced EGF- or PDGF-induced mitogenesis in the presence of IFN- γ . Gil and co-workers²³ reported that IFN- γ greatly enhanced macrophage colony-stimulating factor-induced proliferation of mononuclear phagocytes isolated from Stat1^{-/-} mice but not wild-type mice. They also reported that IFN- γ alone caused a significant growth response of mononuclear phagocytes from Stat1^{-/-} mice but not from wild-type mice. Thus, our findings that IFN- γ exerts a proliferative effect on Stat1^{-/-} lung fibroblasts and enhances the mitogenic effects of growth factors are consistent with other reports that used Stat1^{-/-} bone marrow-derived macrophages or Stat1^{-/-} mouse embryonic fibroblasts.^{23,24}

The mechanism of IFN- γ -induced proliferation of Stat1^{-/-} lung fibroblasts could be due to enhanced phosphorylation of intracellular signaling intermediates or to the induction of autocrine growth factors. We found that the mitogenic effect of IFN- γ on Stat1^{-/-} lung fibroblasts, as well as the ability of IFN- γ to enhance EGF- or PDGF-stimulated growth, was ERK-independent, because IFN- γ neither stimulated ERK phosphorylation nor

increased EGF- or PDGF-stimulated ERK phosphorylation in these cells.

The mitogenic effect of IFN- γ on Stat1^{-/-} cells could be due to Stat1-independent signaling pathways that lead to the autocrine production of promitogenic mediators. Oligonucleotide array analysis has been used to demonstrate a variety of genes that are induced by IFN- γ in Stat1^{-/-} mouse embryonic fibroblasts, including genes encoding secreted growth factors such as HB-EGF and PDGF-AA.²⁵ The concept that IFN- γ induces the release of promitogenic factors has been established. For example, IFN- γ stimulates the release of PDGF-B chain from alveolar macrophages.⁸ IFN- γ also stimulates the release of transforming growth factor- α in human colonic epithelial cells resulting in phosphorylation of the EGF receptor.²⁶ Importantly, up-regulation of these factors favors the development of pulmonary fibrosis after lung injury. We investigated PDGF-AA as a possible mitogenic intermediate in IFN- γ -induced proliferation of Stat1^{-/-} lung fibroblasts. PDGF-AA is a major mitogen produced by lung fibroblasts in response to several cytokines, including interleukin-1 β and interleukin-13.^{3,27} Using quantitative reverse transcriptase-polymerase chain reaction we found that IFN- γ induced PDGF-AA more than twofold in wild-type mouse lung fibroblasts in a time-dependent manner, but IFN- γ only marginally increased PDGF-AA mRNA expression in Stat1^{-/-} mouse lung fibroblasts. Moreover, anti-PDGF-AA neutralizing antibodies had no effect on blocking IFN- γ -induced proliferation of Stat1^{-/-} cells. Therefore, it is not likely that PDGF-AA mediates IFN- γ -induced proliferation of Stat1^{-/-} fibroblasts.

IFN- γ could induce mitogenic mediators other than polypeptide growth factors. Gil and colleagues²³ reported that arginase was inducible by IFN- γ in Stat1^{-/-} bone marrow-derived macrophages. Arginase catalyzes the hydrolysis of arginine to ornithine, which is required for cell proliferation.²⁴ Although Ramana and co-workers²⁵ found arginase induced by IFN- γ in bone marrow-derived macrophages, this same group of investigators did not observe IFN- γ -induced arginase in mouse embryonic fibroblasts. Therefore, although arginase could be important for IFN- γ induced proliferation of Stat1^{-/-} monocytes, it is not a likely candidate mitogen for fibroblasts. In general, it is possible that IFN- γ stimulates Stat1^{-/-} cell proliferation through an autocrine mechanism involving the release of a soluble mitogen. The identity of such an autocrine mediator has not yet been established.

A newly emerging concept is that IFN- γ causes mitogenesis via Stat1-independent signaling pathways, while the growth inhibitory effects of IFN- γ are mediated via Stat1-dependent signaling.²⁸ It is likely that Stat1^{-/-} mice are susceptible to pulmonary fibrosis due to a disrupted growth arrest signaling pathway, which normally mask the promitogenic signals provided by IFN- γ through Stat3 and perhaps other signaling pathways. We found that IFN- γ induced the phosphorylation of Stat3 in both wild-type and Stat1^{-/-} lung fibroblasts. Additionally, IFN- γ -induced Stat3 phosphorylation was increased twofold in Stat1^{-/-} cells compared to wild-type cells. This

observation suggests that the proliferative effect of IFN- γ in Stat1^{-/-} cells could be due in part to enhanced activation of prosurvival factors such as Stat3. Alternatively, the diminished ability of lung fibroblasts from Stat1^{-/-} mice to undergo apoptosis could also be a contributory factor. Kumar and colleagues²⁹ reported defective tumor necrosis factor- α -induced apoptosis in Stat1^{-/-} cells due to low constitutive levels of caspases.

Our findings using Stat1^{-/-} mice could explain why IFN- γ has been of limited value as a therapeutic agent for the treatment of pulmonary fibrosis. Although IFN- γ mediates growth arrest, it also stimulates growth responses and perhaps the production of profibrotic mediators through Stat1-independent signaling pathways. IFN- γ has been reported to have either anti-fibrotic or profibrotic activity in rodent models of lung fibrosis. Gurujeyalakshmi and co-workers³⁰ showed that IFN- γ inhibits pulmonary fibrosis. This anti-fibrotic activity is likely due to the anti-proliferative effects of IFN- γ on fibroblast growth and the ability of IFN- γ to down-regulate transforming growth factor- β production by lung cells. In contrast, Segel and co-workers³¹ reported decreased fibrosis in IFN- γ knockout mice compared to wild-type mice, suggesting that endogenous IFN- γ could play a profibrotic role in bleomycin-induced lung injury. In a clinical trial with idiopathic pulmonary fibrosis patients, a promising beneficial effect of IFN- γ treatment was initially reported.⁹ However, subsequent clinical trials have shown that IFN- γ can cause acute respiratory failure in end-stage pulmonary fibrosis.³² In some other fibroproliferative diseases such as idiopathic myelofibrosis, IFN- γ appears to be ineffective.³³ Further, IFN- γ elicits arteriosclerosis in mice by acting on vascular smooth muscle cells to potentiate growth factor-induced mitogenesis.³⁴

Stat1 deficiency may also have important implications for antecedent viral infection and pulmonary fibrosis. Although the therapeutic application of IFNs may be questionable for the treatment of pulmonary fibrosis, IFNs have potent anti-viral activity. Many viruses evade host defense and the anti-viral effects of IFNs by inactivation of Stat1.³⁵ For example, certain viruses inhibit IFN signaling by targeting Stat1 for proteasome-mediated degradation.³⁶ This may explain why the primary defect of Stat1^{-/-} mice is susceptibility to microbial infections.^{11,12} Although still controversial, there is growing evidence that viral infections exacerbate fibrotic lung diseases. Therefore, Stat1 is likely to play important protective roles in fibrotic lung diseases that are accompanied by viral infection through mediating the anti-viral effects of IFNs and by mediating growth arrest of lung fibroblasts.

In addition to increases in the severity of bleomycin-induced fibrotic lesions and lung collagen content, a greater degree of inflammation was found in Stat1^{-/-} mice compared to wild-type mice. The enhanced inflammatory response of Stat1^{-/-} mice may contribute to proliferation of lung fibroblasts *in vivo*. For example macrophages are major producers of growth factors (PDGF) and mediators (transforming growth factor- β , tumor necrosis factor- α , interleukin-1 β) involved in the fibrotic response. Release of cytokines from other cells such as neutrophils and lymphocytes may also stimulate release

of growth factors to further stimulate fibroblast proliferation. Additionally, IFN- γ is produced by activated macrophages. Given the enhanced mitogenic effect of IFN- γ on Stat1^{-/-} fibroblasts, an increase in inflammatory cells and the profibrotic or promitogenic mediators they release would further augment bleomycin-induced fibrosis in Stat1^{-/-} mice.

The data presented in this study indicates that Stat1 is protective against the progression of lung fibrosis after bleomycin injury which is due to the growth arrest/apoptosis function of this transcription factor. Moreover, our data indicate that IFN- γ exerts a promitogenic effect on fibroblasts in the absence of Stat1. To our knowledge, this is the first study that establishes a definitive role for Stat1 in pulmonary fibrosis. We anticipate that these findings will shed light on the dual actions of IFN- γ in the promotion or inhibition of a fibroproliferative response.

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