TABLE 6. CORRELATION OF DURATION OF DISEASE WITH CLINICAL PROGRESSION AND PULMONARY FUNCTIONS IN PATIENTS WITH AUTOIMMUNE PULMONARY ALVEOLAR PROTEINOSIS

Characteristic	Duration of Disease* ≤1 yr (*n* = 124)	>1 yr, ≤10 yr (*n* = 89)	>10 yr (*n* = 10)	*P* Value†
Disease severity score				0.11
1, % of patients	29.84	20.22	30.00	
2, % of patients	28.23	25.84	20.00	
3, % of patients	22.58	26.97	40.00	
4, % of patients	14.52	20.22	10.00	
5, % of patients	4.84	6.74	0.00	
Pulmonary function				
FVC, % predicted	89.7 ± 18.4	86.6 ± 18.9	80.0 ± 26.2	0.10
FEV_1/FVC	84.5 ± 12.0	84.6 ± 10.6	80.4 ± 8.4	0.12
VC, % predicted	91.0 ± 19.0	87.6 ± 19.4	86.5 ± 23.5	0.08
D_{LCO}, % predicted	71.5 ± 27.7	64.3 ± 24.9	72.1 ± 26.0	0.26
Symptomatic, %	65.0	72.4	70.0	0.28
Disease progression‡				
Symptomatic individuals	(n = 50)	(n = 47)	(n = 8)	0.002
Improved, n	11	20	1	
Worsened, n	6	14	5	
No change, n	33	13	2	
Asymptomatic individuals§	(n = 15)	(n = 21)	(n = 3)	0.99
Improved, n	5	4	2	
Worsened, n	1	2	0	
No change, n	9	15	1	

Definition of abbreviation: D_{LCO} = carbon dioxide diffusing capacity.

* Data are for all participants at enrollment and are presented as percent, median (interquartile range), or mean ± SE.

† Values shown are calculated using the Spearman correlation test.

‡ Treating physician's clinical impression of the severity of pulmonary disease progression from the time of onset of symptoms or an abnormal chest radiograph to the time of enrollment in the study.

§ None of the individuals who were asymptomatic at registration had previously received whole-lung lavage or granulocyte/macrophage colony-stimulating factor aerosol therapy.

the largest contemporaneous cohort of patients with autoimmune PAP assembled to date.

Several lines of evidence support the use of the term "autoimmune PAP" and the stratification of PAP into autoimmune and secondary forms. First, underscoring the importance of GM-CSF in pulmonary surfactant homeostasis, mice deficient in GM-CSF (6, 7) or its receptor (35) develop a pulmonary phenotype biochemically, histologically, physiologically, and ultrastructurally identical to autoimmune PAP in humans (36). Second, GM-CSF autoantibodies seem to be critical to the pathogenesis of autoimmune PAP because high levels are strongly associated with it but are not present in secondary or congenital PAP, other lung diseases, or in healthy individuals (17). Their binding affinity for GM-CSF (~20 pmol/L) is higher than the GM-CSF receptor in its low- (~3,200 pmol/L) or high-affinity (120 pmol/L) binding state (37), and they eliminate GM-CSF bioactivity *in vivo* (18). Third, transfer of purified GM-CSF autoantibodies from patients with PAP into blood from healthy individuals reproduces the myeloid cell abnormalities observed in patients with autoimmune PAP (38). Fourth, anti-murine GM-CSF antibodies reproduce these abnormalities in wild-type mice. Fifth, the courses of autoimmune and secondary PAP are different: Secondary PAP has a far worse outcome (H. Ishii, in preparation).

Important findings of this study are the incidence and prevalence data for autoimmune PAP in Japan. Using an intensive screening approach involving 98% of pulmonary physicians in one region, the highest incidence and prevalence estimates were 0.49 and 6.2 cases per million, respectively. Our prevalence value is higher than reported for Israel (3.7 per million) (39) and lower than for the United States (~10–40 cases per million) (40). One of 15 cases in the former study was congenital PAP, and the latter study included all three clinical forms. If we included all types of PAP identified in Japan during the study period, the prevalence would be 8.7 per million. We did not observe familial clustering in our study, in contrast to the Israeli report in which PAP occurred in two siblings. These likely represent congenital PAP, which occurs secondary to mutations in the genes encoding SP-B (41), surfactant protein C (42), or ABCA3 transporter protein (43). Of the patients with autoimmune PAP in our study, 31% were asymptomatic and were identified by annual medical screening programs. Our observations suggest that the true incidence and prevalence of autoimmune PAP is higher than reported and show that nearly a third of cases are subclinical. Although regional differences in may exist, more studies are needed.

The demographics of Japanese patients with autoimmune PAP differ in several respects from a retrospective meta-analysis done by Seymour and colleagues, which includes most or all cases of PAP reported in the medical literature as of 2002 (2). First, the median age at diagnosis was 51 years and similar in men and women, in contrast to Seymour and colleagues' report in which the median age was 39 years and was different in men and women (39 and 35 years respectively). Second, the age at diagnosis was normally distributed in women and did not have the bimodal pattern previously reported (2). Third, the male:female ratio (2.10:1.0) and, fourth, the proportion of current smokers (28.5%) were lower than previously reported (2.65:1.0 and 72%, respectively) (2). The absence of a male predominance among nonsmokers (never- and ex-smokers) in our study (male:female ratio = 0.60:1.0) is similar to the prior report (0.69:1.0) (2) and is consistent with the possibility that the high proportion of men among patients with PAP may be explained by their higher frequency of tobacco use. However, a high proportion of women in our cohort had no history of smoking (83%) or occupational exposure (87%), suggesting that another factor may be involved in the etiology of autoimmune PAP. Our study did not address potential effects of passive smoke exposure.

It is surprising that COPD was not recognized more commonly in our cohort given that the proportion of current, ex-, and never-smokers was similar to the Japanese population in whom COPD occurs in 8.6% (44, 45). Using the same criterion (FEV_1/FVC < 0.70) as Fukuchi and colleagues (44), only five individuals (2.7%) in our cohort had airflow limitation, whereas 24 individuals were expected of having airflow limitation. Of these, two were male (one ex-smoker, aged 55 yr; one never-smoker, aged 71 yr), and three were female (one current smoker, aged 39 yr; one ex-smoker, aged 28 yr; and one never-smoker, aged 44 yr). Although the reason for this is not clear, it is interesting that asthma, another common lung disorder with an inflammatory component of pathogenesis, was also underrepresented in our cohort (observed frequency = 2.4%; expected frequency ~8.2%). PAP may alter the phenotype of disorders with an inflammatory component of the pathogenesis. This is supported by observations that GM-CSF is required for myeloid cell functions in humans (38) and mice (9), where it regulates a number of innate immune responses, including the TLR4 response to lipopolysaccharide (46), and GM-CSF autoantibodies in patients with PAP virtually eliminate GM-CSF bioactivity (18). Thus, GM-CSF autoantibodies may blunt inflammatory responses in patients with PAP, which may affect tissue destruction in COPD and the tendency for exacerbations in asthma.

The DSS (22) provided a useful measure of lung disease severity in symptomatic and asymptomatic autoimmune patients with PAP, which was important because nearly one-third of the patients were asymptomatic and because dyspnea is difficult to quantify in PAP due to the insidious onset. A limitation of this

study was the absence of a dyspnea index. Notwithstanding, the DSS correlated well with the D_{LCO} % predicted, less well with FVC % predicted and VC % predicted, and not with FEV_1/FVC. Although PAP is usually described as a restrictive lung disease, reductions in lung volumes in autoimmune PAP were minor and fell in the normal range in most patients, suggesting that these pulmonary function measures may be of limited usefulness in assessing the severity of PAP lung disease. Physiologically insignificant restriction is further supported by the absence of hypoventilation, even in severe cases. Thus, in autoimmune PAP, hypoxemia is primarily due to reduced oxygen diffusion and possibly ventilation–perfusion mismatching.

Infections were less common among Japanese PAP registrants than previously reported (2). Furthermore, although *Nocardia* was identified in 60% of reported PAP cases complicated by infection (2), no cases of *Nocardia* infection were observed in our study during the period of observation. It is possible that these differences represent reporting bias or differences in clinical care of early infections because a number of the prior reports reflect infectious complications occurring over four decades.

Our observation that GM-CSF autoantibody levels did not correlate with disease severity as measured by the presence of symptoms, pulmonary function testing, or the DSS is consistent with prior reports (2, 3, 22). Because GM-CSF autoantibodies in patients with PAP are polyclonal, it is possible that measuring the level of neutralizing antibody may provide a better correlation with disease severity. We have reported a patient with autoimmune PAP in whom serial measurement of serum GM-CSF neutralizing activity correlated well with disease severity (47). Furthermore, the serum GM-CSF neutralizing activity was reduced in a patient who was successfully treated with inhaled GM-CSF (47). Pulmonary compartmentalization of GM-CSF antibodies may be important in determining disease severity and could explain the lack of correlation with serum autoantibody levels. Neither the autoantibody levels, proportion of symptomatic individuals, pulmonary functions, nor DSS correlated with the duration of disease, which is consistent with the concept that disease severity does not worsen with time in most patients.

The method used to measure GM-CSF autoantibody levels is similar to prior reports (17) except that a new monoclonal GM-CSF autoantibody standard was used. Although this standard yields reproducible results, it yields autoantibody levels one seventh that of the previous GM-CSF autoantibody standard isolated from pooled PAP serum (3, 15, 17, 19) and similar in sensitivity and specificity. Our results support the use of GM-CSF autoantibody measurement in the diagnosis of autoimmune PAP as an adjunct to chest CT and bronchoscopy.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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APPENDIX

The patients were referred to one of the nine primary clinical research centers by the following physicians (only one individual is listed per institution because of space limitations): Arata Azuma (Nippon Medical School), Masato Katagiri (Kitazato University), Masafumi Niijima (Narita Red Cross Hospital), Shinobu Akagawa (NHO Tokyo Hospital), Masayuki Nara (Tohoku University), Akira Fujita (Metropolitan Fuchu Hospital), Ryo Takahashi (Osaka Prefectural Medical Center for Respiratory and Allergic Diseases), Jun Sato (Hamamatsu University School of Medicine), Hidenori Ichiyasu (Kumamoto University), Yoshihiro Honda (Sendai Kohsei Hospital), Yoshio Taguchi (Tenri Hospital), Masakazu Aitani (NTT West Osaka Hospital), Masanori Nakanishi (NHO Tsuruga Hospital), Tetsuo Yamaguchi (JR Tokyo General Hospital), Muneharu Maruyama (Toyama University Hospital), Atsuhiro Fujii (Juntendo University), Kohei Yamauchi (Iwate Medical University), Towako Nagata (Nagasaki University), Tatsuro Mikawa (Yodogawa Christian Hospital), Toshihiko Hashizume (Yokohama Kyosai Hospital), Sakae Honnma (Toranomon Hospital), Masato Tohyama (University of the Ryukyus), Masaharu Nagayama (Shizuoka City Hospital), Noriharu Shijubo (Sapporo Medical University), Koichiro Takahashi (Saga Medical University), Iwao Komuro (Metropolitan Hiroo Hospital), Mihoko Doi (Hiroshima University), Kaoru Maki (Matsue Seikyo Hospital) Yoshitsune Sando (Gunnma University), Hiroo Miyazaki (Fukuroi Municipal Hospital), Youkou Shibata (Yamagata University), Hirohisa Toga (Kanazawa Medical College), Naotoshi Suruta (NHO Wakayama Hospital), Hiroaki Kume (Nagoya University), Ken Nawa (Hitachi General Hospital), Kaneo Kawazoe (Naka Tsushima Hospital), Watako Takehara (Chubu Rosai Hospital), Yasuhiro Ieda (Kinki University Sakai Hospital), Masaru Yauchi (Ishimaki Red Cross Hospital), Yuji Akiba (Asahikawa Kosei Hospital), Masako Toh (The Fraternity Memorial Hospital), Toshiyuki Yamauchi (Keihai Rosai Hospital), Yuzuru Inoue (Shin Yamate Hospital), Kenji Kohno (Kyoto Prefectural University of Medicine), Machiko Arita (Kurashiki Central Hospital), Kazunari Himeno (Fujita Health University), Nobuto Kishimoto (Takamatsu Municipal Hospital), Masaya Yamasato (NHO Minami Yokohama Hospital), Aya Sugawara (Fukishima Medical University), Atuko Kobayashi (Saiseikai Suita Hospital), Katsunori Sugisaki (Oita University), Kenichiro Ohtani (Osaka City University Medical School), Yoshikazu Ishii (Jichi Medical University), Yoshiki Kobayashi (Takatsuki Red Cross Hospital), Shigeru Koyama (Nagano Red Cross Hospital), Hiroko Kimura (Tohoku Rosai Hospital), Atuhiro Goto (Okazaki City Hospital), Amihiko Hirano (Wakayama Medical University), Jun Shiraki (Kochi Health Science Center), Fumiko Sugatani (Teine Kijinkai Hospital), Akira Miyashita (Yokohama City University School of Medicine), Momoyo Ukai (Tokushima University), Yoshida Makino (Osaka Medical College), Hidenori Mori (Gifu University), Susumu Oguri (NHO Minami Kyoto Hospital), Taku Inoue (Sano Kousei Sougou Hospital), Masaaki Takahashi (Asahikawa Medical University), Michihiro Yoshimi (Kyusyu University), Toshiaki Hidaka (Koga Sogo Hospital), Masahiko Iwaoka (Fujieda Municipal Central Hospital), Daizen Cho (Tsubame Rosai Hospital), Eishi Ito (Hakodate City Hospital), Hiroyo Okurakata (Saiseikai Sanjo Hospital), Hiroshi Saiki (NHO Miyazaki Hospital), Jun Katsuta (Takayama Red Cross Hospital), Yoshifumi Imazu (Miyazaki University), Mikiko Ono (Kagoshima University), Tatsuya Hosono (Jichi Medical College), Shinji Takeuchi (Takamatsu Red Cross Hospital), Kenji Konishi (Seirei Hamamatsu General Hospital), Mikio Oka (Kawasaki Medical University), Takefumi Saito

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Overproduction of collagen and diminished SOCS1 expression are causally linked in fibroblasts from idiopathic pulmonary fibrosis

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, and often fatal pulmonary disorder, and its pathology is characterized by parenchymal fibrosis. To investigate the characteristics of fibroblasts in IPF, we obtained eight fibroblast cell lines from lungs with IPF and eight lines from normal lungs. We found that the fibroblasts from IPF spontaneously produced higher amounts of type I collagen and had lower expression levels of SOCS1 than fibroblasts from normal lung. By using mouse fibroblasts, we demonstrated the causal relationship between them: the deficiency of SOCS1 in fibroblasts resulted in increased collagen production, whereas overexpression of SOCS1 suppressed collagen production. IFN-γ suppressed spontaneous collagen production even in SOCS1-deficient fibroblasts, indicating that IFN-γ inhibition is SOCS1-independent. In contrast, IFN-γ suppressed the increase of collagen production induced by IL-4 in wild type fibroblasts but not SOCS1-deficient fibroblasts, suggesting IFN-γ acted exclusively via SOCS1 in this case. Following IFN-γ stimulation, the amount of SOCS1 mRNA expressed by IPF fibroblasts was comparable to that of normal fibroblasts. Thus, the extent of SOCS1 increase after stimulation by IFN-γ was significantly higher in IPF fibroblasts. The extent to which IFN-γ inhibited collagen production was also larger in IPF fibroblasts than in normal fibroblasts. These results suggest that the exaggerated production of collagen observed in fibroblasts from IPF is causally related to the diminished expression of SOCS1, and IPF fibroblasts are more susceptible to IFN-γ because of decreased expression of SOCS1.

Keywords: Idiopathic pulmonary fibrosis; IFN-γ; SOCS1; Fibroblasts; Collagen

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, and often fatal pulmonary disorder. It is characterized by parenchymal fibrotic changes and by worsening respiratory symptoms and gas exchange. No therapy has been proved to be beneficial [1]. Recently, it was reported that interferon-gamma (IFN-γ) may have a potential therapeutic role in the management of IPF. A small randomized controlled trial revealed that the combination of IFN-γ1b and prednisolone significantly improved physiological parameters in IPF patients compared with prednisolone alone [2]. However, another randomized controlled trial showed that IFN-γ1b did not affect pulmonary function or the quality of life [3]. A recent meta-analysis revealed that IFN-γ1b therapy was associated with reduced mortality in patients with IPF [4].

Beneficial effects of IFN-γ on fibrotic lung diseases had been suggested by many *in vitro* studies. There is an imbalance between Th1 and Th2 responses in favor of Th2 in lungs of IPF, which leads to the accumulation of collagen [5–7]. Th2 cytokines including IL-4 can promote fibroblast activation and fibrosis [8]. It has been proposed that inhibition of Th2 cytokine signaling could be used therapeutically to reduce fibrosis [9,10]. The Th1 cytokine, IFN-γ, can

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inhibit proliferation of fibroblasts and fibroblast-mediated angiogenesis. It also decreases the expression of both procollagen mRNA and the growth mediator transforming growth factor (TGF)-β in fibroblasts [11]. IFN-γ down-regulates the transcription of the gene for TGF-β and IL-4 in the bleomycin-induced model of lung fibrosis [12–14]. In addition, adoptive transfers of Th1 clones can diminish peribronchial fibrosis in a murine model of asthma [15]. However, the effects of IFN-γ on fibroblasts from IPF patients have not been examined.

Suppressor of cytokine signaling 1 (SOCS1) was cloned in 1997 [16–18]. SOCS1 is normally expressed at very low levels, but many cytokines, especially IFN-γ, induce SOCS1 expression [18,19]. SOCS1 binds JAK2 and interrupts IFN-γ signaling by inhibiting activation of STAT1 [20,21]. These results indicate that SOCS1 constitutes a negative feedback system. SOCS1 also binds JAK1 and JAK3 and interrupts IL-4 signaling by inhibiting phosphorylation of STAT6. Because IPF is a Th2 cytokine dominant disease, SOCS1 may suppress collagen production by inhibiting Th2 cytokine signaling. In addition, the inhibitory effect of IFN-γ on collagen production may be mediated by SOCS1.

In this study, we investigated both the expression of SOCS1 and spontaneous collagen production in lung fibroblasts from IPF patients and normal fibroblasts. To explore the causal relationship between expressions of SOCS1 and collagen production in fibroblasts, we employed murine fibroblasts from SOCS1-deficient and wild type mice, and their stable SOCS1 transfectants. Furthermore, we compared the effects of IFN-γ on IPF and normal fibroblasts.

Materials and methods

Mouse embryonal fibroblasts and preparation of human lung fibroblasts. Normal human lung fibroblasts were established from lung cancer patients (normal fibroblasts; $n = 8$) from whom the tissues were taken from a site distal to the tumor. Fibrotic human lung fibroblasts were established from the tissues of open lung biopsies performed in the usual interstitial pneumonia (UIP) form ($n = 8$). The use of all clinical materials was conducted with written informed consent and was approved by the Research Ethics Committees of Hiroshima University. Wild type mouse embryonal fibroblasts and SOCS1-deficient mouse embryonal fibroblasts were obtained as described previously [22].

Culture medium and cytokines. All fibroblasts were cultured in appropriate media supplemented with 10% heat-inactivated FBS (Biowest, Nuaillé, France) and were maintained at 37 °C in an atmosphere of humidified air with 5% CO_2. For assays, all fibroblasts were cultured without FBS. Mouse and human recombinant IL-4 and IFN-γ, and human recombinant TGF-β1 (R&D Systems, Minneapolis, USA) were used.

ELISAs. Mouse embryonal fibroblasts (1.5×10^5) were grown for 24 h in 6-well dishes. After serum starvation for 12 h, spent medium was replaced with fresh medium with or without cytokines. Human lung fibroblasts (5.0×10^4) were grown for 3d in 24-well dishes. After washing, fresh medium was added. Forty-eight-hour later, the supernatants were collected. Mouse and human type I collagen were measured by ELISA systems. Samples and standards were coated directly in Nunc MaxiSorp™ flat-bottom 96-well plates (Cat No. 44-2404; NALGE NUNC International, Rochester, NY) overnight at 4 °C. After blocking, polyclonal anti-mouse type I collagen antibody (BioDesign, Saco, ME) or mouse anti-human collagen type I monoclonal antibody (Chemicon International, Temecula, CA) was added. Polyclonal HRP-conjugated goat anti-rabbit Ig antibody or anti-mouse Ig antibody (GE Healthcare Bio-sciences, Piscataway, NJ) was used to measure binding of primary antibodies.

Western blot analysis. Cells were treated with lysis buffer (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 10 mM Na_2VO_4, protease inhibitor cocktail, and 1 mM PMSF) and clarified by centrifugation. We used an ECL Western-blotting analysis system (GE Healthcare Bio-sciences, Piscataway, NJ), as previously described [23]. SDS–PAGE was performed and PAGE-separated proteins were electroblotted onto nitrocellulose membranes (GE Healthcare Bio-sciences) and incubated with anti-phospho-specific STAT6 monoclonal antibody (Tyr641; Cell Signaling Technology) or anti-myc antibody (4A6; Upstate, NY, USA). A goat anti-rabbit IgG-HRP antibody or anti-mouse IgG antibody (GE Healthcare Bio-sciences) served as the secondary antibodies for these experiments. After the antibodies had been stripped, the filters were reprobed with anti-STAT6 polyclonal antibody (R&D Systems). As internal controls, monoclonal anti-α-tubulin (DM 1A; Sigma, St. Louis, MO) was used.

Reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time quantitative RT-PCR. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Tokyo, Japan) following the manufacturer's instructions. Equivalent amounts of total RNA (0.5 μg) were primed with oligo(dT), and first strand cDNA was synthesized by using Revatra Ace (TOYOBO, Osaka, Japan), according to the manufacturer's protocol. The PCR primers were as follows: mouse *SOCS1*, 5′-CACCTTCTTGGTGC GCGACA-3′ (forward) and 5′-GCAGCTCGAAAAGGCAGTCG-3′ (reverse); mouse *β-actin*, 5′-GTGATGGTGGGAATGGGTCAG-3′ (forward) and 5′-TTTGATGTCACGCACGATTTCC-3′ (reverse). human *SOCS-1*, 5′-TTGGAGGGAGCGGATGGGTGTAG-3′ (forward) and 5′-GCAGCTCGAAAAGGCAGTCG-3′ (reverse); human *β-actin*, 5′-GT GGGGCGCCCCAGGCACCA-3′ (forward) and 5′-GCTCGGCCGTG GTGGTGAAGC-3′ (reverse). Semi-quantitative RT-PCR and real-time quantitative PCR were optimized for the number of cycles to ensure product intensity within the logarithmic phase of amplification.

Cloning of SOCS1 cDNA and establishment of fibroblasts stably expressing SOCS1. The entire coding region of *SOCS1* was amplified by RT-PCR. The product was cloned into a pcDNA3.1-myc/His A(+) vector (Invitrogen, Carlsbad, CA) (pcDNA3.1-*SOCS1*-myc/His). The SOCS1-myc cording sequence was obtained by PCR amplification. The PCR product was inserted into a pCLXSN retroviral vector (pCLXSN-*SOCS1*-myc). The sequence of cloned SOCS1 cDNA was confirmed with TOYOBO Gene Analysis (Tsuruga, Japan). pCLXSN-*SOCS1*-myc or pCLXSN (control) was transfected to 293 cells with pCL-Eco. Embryonic fibroblasts were incubated with viral supernatant. The wild type and SOCS1-deficient murine fibroblast transformants expressing myc-tagged SOCS1 were selected with 1.0 mg/mL G418 (Promega, Madison, USA). Expression of SOCS1 was determined in each clone by RT-PCR, Western blotting and immunostaining.

Statistical analysis. The results are expressed as means ± SD. The statistical significances of differences were analyzed by the Mann–Whitney *U* test or Student's *t* test. The differences were considered significant if the probability value was <0.05.

Results and discussion

Human lung fibroblasts obtained from IPF patients produced higher amounts of type I collagen and expressed lower levels of SOCS1 mRNA than those from normal lungs

We obtained eight fibroblast lines from normal lungs and 8 from fibrotic portion of IPF lungs. The fibroblasts from IPF patients (IPF fibroblasts) produced significantly higher amounts of type I collagen than did fibroblasts from

normal lung (normal fibroblasts) as determined by ELISA methods ($p = 0.046$; Fig. 1a). The IPF fibroblasts also had higher levels of pro-alpha1-(I) collagen mRNA expression (data not shown). These results confirmed previous observations that the production of collagen from IPF fibroblasts is elevated when compared with normal fibroblasts [24,25].

Since we hypothesized that SOCS1 may be involved in over-production of collagen from lung fibroblasts, we examined SOCS1 mRNA expression levels in our human fibroblasts. Using a real-time PCR method, IPF fibroblasts showed significantly lower expression levels of SOCS1 mRNA than did normal fibroblasts ($p = 0.0012$; Fig. 1b).

SOCS1-deficient fibroblasts produced higher amounts of type I collagen than wild type fibroblasts

To prove the causal relationship between reduced expression of SOCS1 and increased production of type I collagen in fibroblasts, we employed murine embryonal fibroblasts (MEF) from SOCS1-deficient mice and their wild type littermates. In wild type MEF, IFN-γ stimulated SOCS1 mRNA expression within an hour and it continued for six hours (Fig. 2a). In contrast, MEF from SOCS1-deficient mouse showed no detectable SOCS1 mRNA under the same conditions.

The SOCS1-deficient MEF spontaneously produced significantly higher amounts of type I collagen than did wild type MEF at 36, 48, and 72 h ($p = 0.004$, $p = 0.001$, $p < 0.001$, respectively; Fig. 2b). These results indicate that SOCS1 deficiency increases spontaneous collagen production in fibroblasts.

Overexpression of SOCS1 suppressed type I collagen production from fibroblasts

To further investigate the association between SOCS1 and collagen production in fibroblasts, we examined the effects of overexpression of SOCS1. First we generated wild type MEF stably expressing myc-tagged SOCS1 protein (SOCS1-myc), and explored the effects on tyrosine phosphorylation of STAT6 induced by IL-4 (Fig. 2c). In the control wild type MEF, STAT6 was tyrosine-phosphorylated in response to IL-4, but overexpression of SOCS1 resulted in almost complete inhibition of STAT6 phosphorylation. This indicates that the introduced SOCS1 was functionally active.

The wild type MEF with SOCS1-myc produced significantly lower levels of type I collagen than did control fibroblasts ($p = 0.008$, Fig. 2d). Then, we generated SOCS1-deficient MEF stably expressing SOCS1-myc. This fibroblast line also produced lower amounts of type I collagen than did control SOCS1-deficient MEF ($p = 0.002$, Fig. 2e). These results indicate that overexpression of SOCS1 can inhibit type I collagen production from fibroblasts.

Taken together with previous observations, we concluded that endogenous production of collagen is causally linked to the level of SOCS1 expression.

IFN-γ mediated suppression of type I collagen production is partially mediated by SOCS1

IFN-γ has anti-fibrotic effects and may have a potential therapeutic role in the management of IPF as described above. It has been reported that IFN-γ inhibits

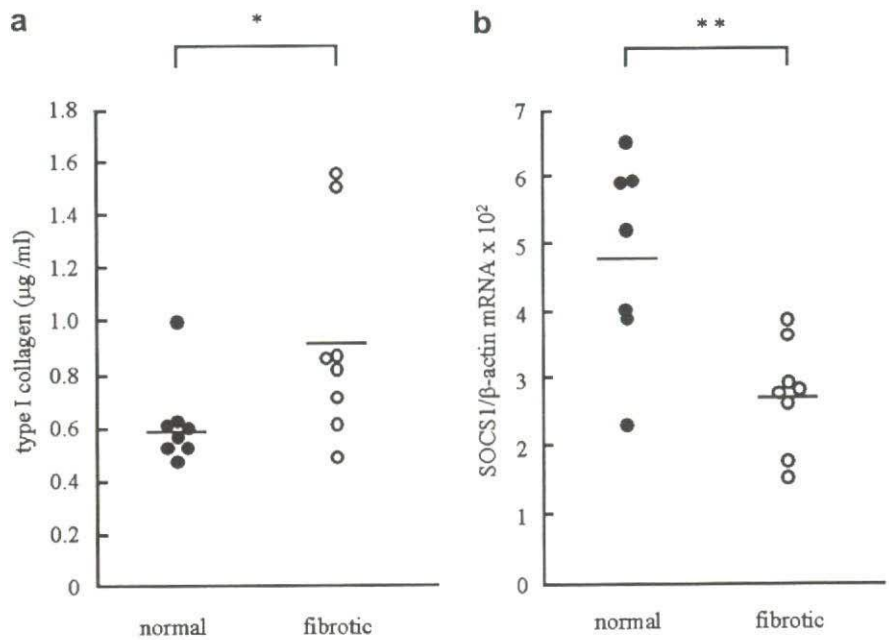

Fig. 1. Fibroblasts from IPF produced higher amounts of type I collagen and expressed lower levels of SOCS1 mRNA than did those from normal lungs. (a) Human lung fibroblasts were cultured for 48 h and the supernatants were collected for ELISA of human type I collagen. (b) Human lung fibroblasts were cultured for 3d, RNA was extracted and cDNA was synthesized. The expression levels of human SOCS1 mRNA were evaluated using real-time quantitative RT-PCR. (Mann–Whitney U test; $^{*}p < 0.05$, $^{**}p < 0.01$.)

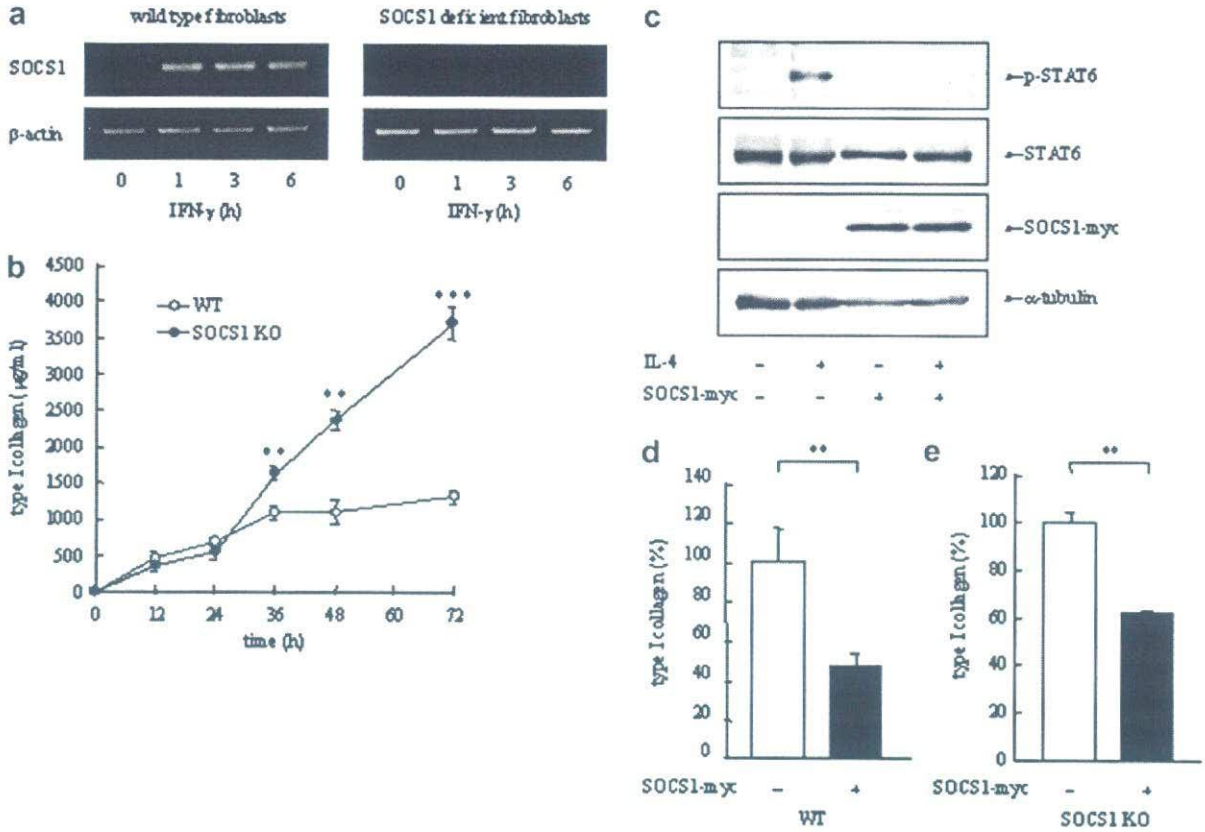

Fig. 2. SOCS1-deficient fibroblasts produced higher amounts of type I collagen than wild type fibroblasts and overexpression of SOCS1 suppressed type I collagen production from fibroblasts. (a) Wild type and SOCS1-deficient mouse fibroblasts were incubated with IFN-γ (50 ng/mL). The expression of SOCS1 mRNA was evaluated by RT-PCR. (b) Wild type and SOCS1-deficient mouse fibroblasts were cultured for the indicated time, and the supernatant was collected for ELISA. The amount of type I collagen was significantly higher in SOCS1-deficient fibroblasts than wild type fibroblasts after 24 h of culture. (c) Exogenous SOCS1 was functionally active. Wild type mouse fibroblasts with SOCS1-myc and control fibroblasts were incubated with or without IL-4 (50 ng/mL) for 2 h. The cell lysates were fractionated by SDS–PAGE and blotted with antibodies to phospho-STAT6, STAT6, myc (4A6), and α-tubulin. (d) Wild type mouse fibroblasts with SOCS1-myc and control fibroblasts were cultured for 48 h. The amount of type I collagen in the supernatant was evaluated by ELISA. The amount of type I collagen is shown as a percentage of those from the control wild type fibroblasts without SOCS1-myc (100% = 1097 μg/mL). (e) Using the same methods, the amounts of type I collagen production from SOCS1-deficient fibroblasts with SOCS1-myc and control fibroblasts were analyzed. The amount of type I collagen is shown as a percentage of those from the control SOCS1-deficient fibroblasts without SOCS1-myc (100% = 2360 μg/mL). Results are shown as means ± SD of values from three experiments. (Student's t test; $^{**}p < 0.01$, $^{***}p < 0.001$.)

TGF-β1- and IL-4-enhanced collagen production in lung fibroblasts in vitro [8,11,26]. To confirm this, we examined the amount of collagen produced by MEF in response to these cytokines. The amount of type I collagen produced by unstimulated wild type MEF was decreased by IFN-γ in a dose-dependent fashion (Fig. 3a). Both TGF-β1 and IL-4 increased collagen production, and their enhancements were completely inhibited by the addition of IFN-γ (Fig. 3c).

In the case of SOCS1-deficient MEF, IFN-γ also inhibited spontaneous production of collagen in a dose-dependent fashion (Fig. 3b). Although SOCS1 mediates some effects of IFN-γ, these results indicate that IFN-γ can suppress spontaneous production of collagen independently of SOCS1. The inhibitory effect of IFN-γ at the highest concentration examined (50 ng/mL) was greater in SOCS1-deficient than wild type fibroblasts (76.7 ± 1.4%, and 58.8 ± 6.5%, respectively; $p = 0.019$). This may be explained by the fact that signaling by IFN-γ cannot be inhibited by endogenous SOCS1 in SOCS1-deficient cells. Since SOCS1 is a strong inhibitor of IFN-γ signal transduction, the deficiency of this molecule would enhance the effect of IFN-γ. Both TGF-β1 and IL-4 increased collagen production in SOCS1-deficient fibroblasts (Fig. 3d). IFN-γ suppressed the enhancement of type I collagen production induced by TGF-β1 but did not significantly suppress the enhancement induced by IL-4 in SOCS1-deficient mouse fibroblasts. These results indicate that the inhibitory effect of IFN-γ on IL-4-induced (but not TGF-β1-induced) enhancement of collagen production is mediated exclusively by SOCS1. Taken together, IFN-γ can suppress collagen production from fibroblasts via both SOCS1-dependent and -independent mechanisms.

Effect of IFN-γ on human lung fibroblasts obtained from IPF

Finally, we examined effects of IFN-γ on IPF fibroblasts. To our knowledge, there had been no previous

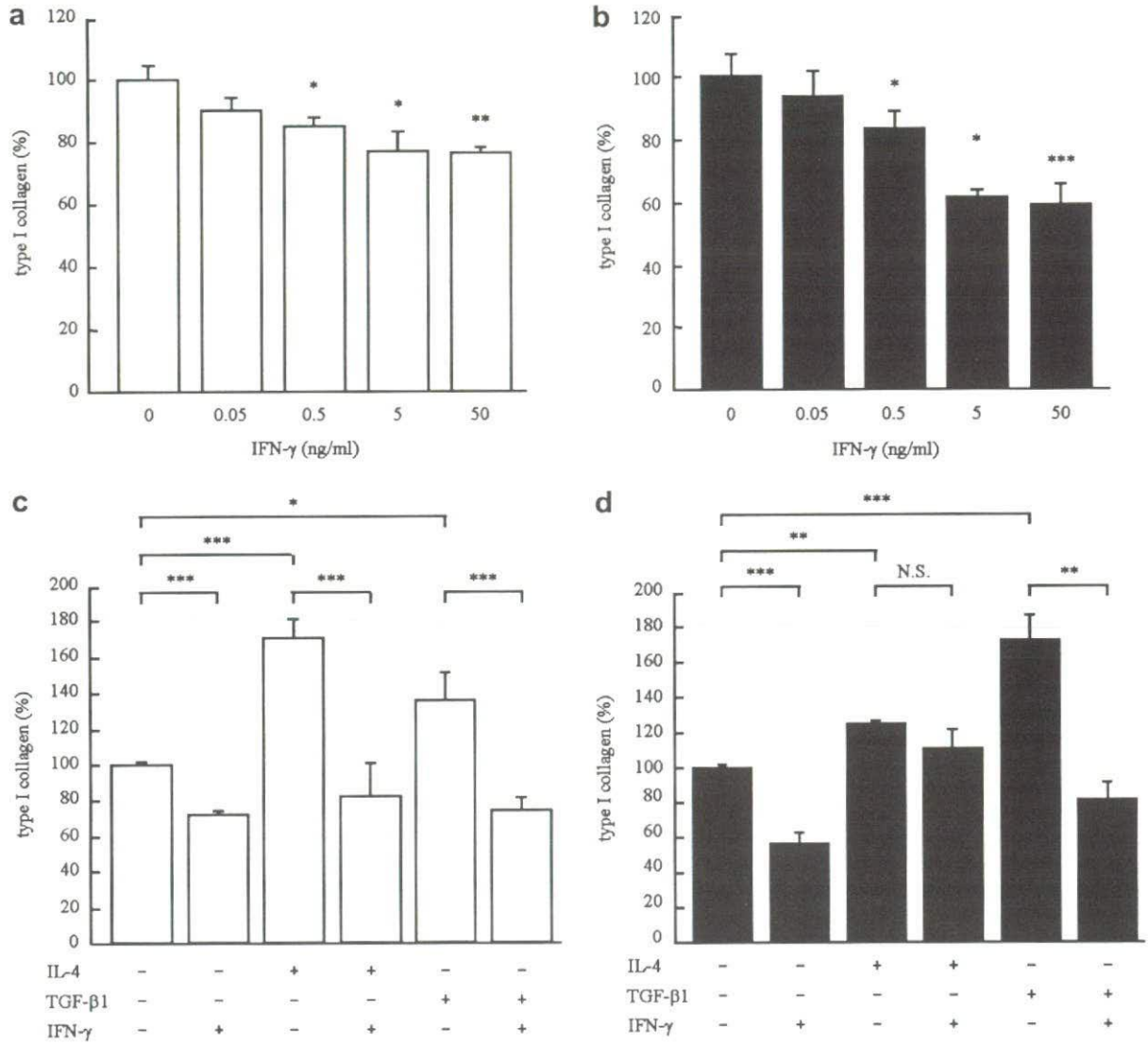

Fig. 3. IFN-γ suppressed type I collagen production and SOCS1 partially mediated the IFN-γ-induced inhibition of collagen production. (a,b) Wild type (a) and SOCS1-deficient mouse fibroblasts (b) were stimulated with indicated concentrations of IFN-γ for 48 h. The amount of type I collagen in the supernatants was evaluated by ELISA. The amount of type I collagen is shown as a percentage of those from fibroblast not stimulated with IFN-γ. (c,d) Wild type (c) and SOCS1-deficient mouse fibroblasts (d) were incubated with or without indicated cytokines for 48 h and the supernatants were collected for ELISA. The amount of type I collagen is shown as a percentage of those from fibroblast not stimulated with cytokines. (Student's t test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.)

reports exploring the effect of IFN-γ on IPF fibroblasts. Following treatment with IFN-γ, IPF fibroblasts expressed the same amount of SOCS1 mRNA as normal fibroblasts (Fig. 4a). Since the IPF fibroblasts expressed lower levels of SOCS1 before stimulation, the extent of increase after stimulation was significantly greater for IPF fibroblasts than for normal fibroblasts ($p = 0.021$; Fig. 4b). IFN-γ inhibited collagen production more effectively in IPF fibroblasts than normal fibroblasts ($p = 0.012$; Fig. 4c), as expected from the observation in SOCS1-deficient fibroblasts.

In the present study, we demonstrated decreased expression of SOCS1 in IPF fibroblasts. The underlying mechanism is not apparent. Decreased expression might have been due to altered endogenous production of cytokines such as IFN-γ or IL-4, which induce SOCS1 in fibroblasts. However, this was not the case because the concentrations of IFN-γ, IL-4 and IL-13 were not different among culture supernatants containing 10% FBS or cultures using serum-free medium (data not shown). Our results are consistent with a previous study showing that deficient expression of SOCS1 is associated with fibrotic changes in the liver [27]. Mice with low expression of the SOCS1 gene ($SOCS1^{-/+}$) developed more severe liver fibrosis. In human hepatitis, severity of liver fibrosis was reported to be well correlated with SOCS1 gene methylation, which results in depressed expression of SOCS1 mRNA. We also investigated the possibility that methylation of the SOCS1 gene was elevated in IPF fibroblasts. However, no support for this theory could be obtained in our fibroblasts (data not

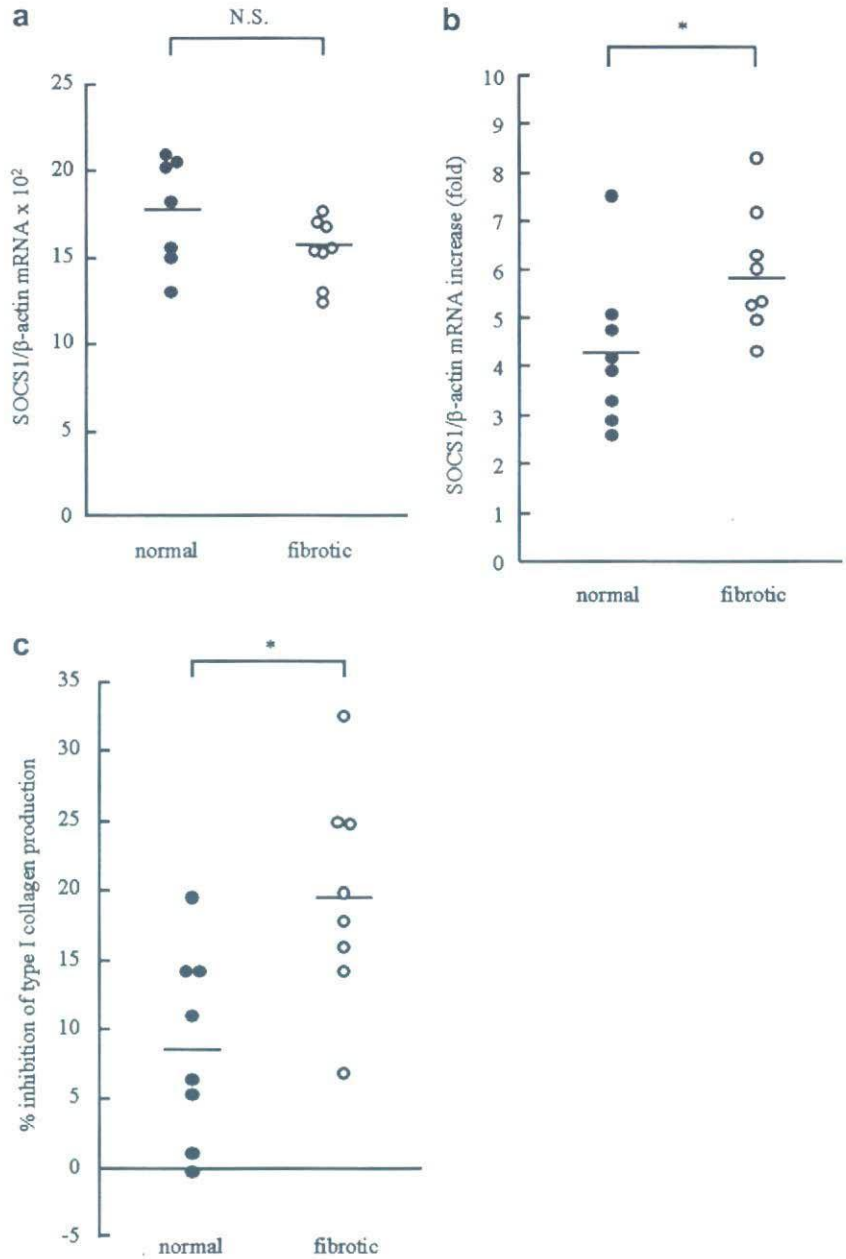

Fig. 4. IFN-γ-mediated induction of SOCS1 and suppression of collagen production were more pronounced for IPF fibroblasts than normal fibroblasts. (a) Normal and IPF human lung fibroblasts were cultured with IFN-γ for 2 h and the expression levels of human SOCS1 mRNA were evaluated by real-time quantitative RT-PCR. (b) The relative increase in the SOCS1/β-actin mRNA ratio after IFN-γ stimulation is indicated as fold-index. The fold-increase in SOCS1 mRNA after IFN-γ stimulation was significantly larger in IPF fibroblasts. (c) IFN-γ-mediated suppression of collagen production was also larger in IPF than normal fibroblasts. Human lung fibroblasts were cultured for 48 h with or without IFN-γ and the amount of type I collagen was evaluated by ELISA. Data are shown as % inhibition of type I collagen production and represented as the mean of three independent experiments. (Mann–Whitney U test; $^{*}p < 0.05$.)

shown). The cause(s) for lower expression of SOCS1 in fibrotic fibroblasts requires further analyses.

The present study of lung fibroblasts from IPF patients suggests that elevated production of collagen is causally related to diminished expression of SOCS1. In addition, we demonstrated that IFN-γ could inhibit collagen production via both SOCS1-dependent and -independent mechanisms. Furthermore, IFN-γ therapy could be more effective with IPF fibroblasts than normal cells, at least in terms of collagen production. The decreased expression of SOCS1 may explain the greater susceptibility to IFN-γ in IPF fibroblasts. The present study provides novel insights into the pathophysiology of fibrotic lung diseases such as IPF.

Acknowledgment

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Suppressor of cytokine signaling 1 inhibits pulmonary inflammation and fibrosis

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Background: Suppressor of cytokine signaling (SOCS) proteins are inhibitors of cytokine signaling. Our previous study suggested that SOCS1 regulates collagen synthesis by lung fibroblasts, suggesting a role of SOCS1 in the pathophysiology of pulmonary fibrosis.

Objectives: We sought to investigate the role of SOCS1 in pulmonary inflammation and fibrosis *in vivo*.

Methods: SOCS1-haplodeficient mice treated with bleomycin (BLM) were evaluated for pulmonary inflammation and fibrosis compared with wild-type mice. The human study group was composed of 18 patients with interstitial lung disease. Lung specimens obtained by means of open lung biopsy were investigated to determine whether the severity of fibrosis was associated with decreased SOCS1 expression. Finally, we further analyzed the effect of exogenous SOCS1 on BLM-induced lung injury based on adenoviral *SOCS1* gene transfer to the lung.

Results: SOCS1-haplodeficient mice treated with BLM showed markedly enhanced pulmonary inflammation and fibrosis compared with wild-type mice. Using human lung specimens, we found that *SOCS1* mRNA levels inversely correlated with duration of the disease. SOCS1 expression was significantly less in lung tissue from patients with idiopathic pulmonary fibrosis (IPF) compared with that in non-IPF patients. Moreover, SOCS1 expression was significantly less in severe fibrotic lesions (lower lobe) than in less fibrotic lesions (upper lobe). Adenoviral *SOCS1* gene transfer to murine lungs significantly decreased lymphocytic inflammation, pulmonary fibrosis, and mortality because of BLM-induced lung injury. Exogenous SOCS1 inhibited expression of various cytokines, including TNF-α, which might play a key role.

Conclusions: These results suggest that SOCS1 might act as a suppressor for pulmonary fibrosis. SOCS1 might be a target of IPF treatment. (J Allergy Clin Immunol 2008;121:1269-76.)

Key words: *Pulmonary fibrosis, Suppressor of cytokine signaling, tumor necrosis factor α, adenoviral vector, bleomycin*

Abbreviations used
BALF: Bronchoalveolar lavage fluid
BLM: Bleomycin
CHP: Chronic hypersensitivity pneumonitis
ILD: Interstitial lung disease
IPF: Idiopathic pulmonary fibrosis
NSIP: Nonspecific interstitial pneumonia
SOCS: Suppressor of cytokine signaling
WT: Wild-type

Pulmonary fibrosis is a disease of known and unknown cause characterized by lung destruction and dysfunction. The typical and most common phenotype is idiopathic pulmonary fibrosis (IPF). There has been no effective therapy for this lethal disease, and the prognosis of IPF is extremely poor.[1] A variety of cytokines and chemokines are involved in its pathophysiology. Of these, T_H2 cytokines are now considered to be dominant.[2]

The animal model most frequently used to study human pulmonary fibrosis is bleomycin (BLM)-induced lung fibrosis in rodents.[3,4] Like human IPF, murine BLM-induced lung injury is associated with the T_H2-dominant cytokine pattern.[2] In addition, we previously demonstrated that adoptive transfer of T_H1 clones leads to reversible alveolitis but not fibrosis.[5] T_H2 cells and T_H2 cytokines might be essential for induction of pulmonary fibrosis because inhibition of the T_H2 cytokines diminished fibrosis in this model.[6]

Suppressor of cytokine signaling (SOCS) family molecules inhibit cytokine signals by regulating the Janus kinase–signal transducer and activator of transcription pathway. SOCS, induced by various cytokines and hormones, regulates not only cytokine signals but also T_H1/T_H2 cell differentiation.[7] At present, there are 8 SOCS molecules; cytokine-inducible SH2 domain–containing protein and SOCS1 to SOCS7. The role of SOCS1 in T_H cell differentiation is not fully understood; however, a previous report suggested that SOCS1 is dominantly expressed in T_H1 cells.[8] In addition, SOCS1 is an inhibitor of profibrotic cytokines, such as IL-4 and TNF-α.[9] Transduction of TNF-α signal can be inhibited by SOCS1 but not by other SOCS proteins.[9] Therefore SOCS1 might suppress pulmonary fibrosis through inhibiting profibrotic cytokines.

We previously demonstrated that higher amounts of type I collagen and lower levels of *SOCS1* mRNA were produced by fibroblasts from lungs of patients with IPF than from healthy lungs. Furthermore, the deficiency of SOCS1 in murine fibroblasts resulted in increased collagen production, whereas overexpression of SOCS1 suppressed collagen production *in vitro*.[10] From these findings, we hypothesized that SOCS1 would be involved in the pathophysiology of lung fibrosis.

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Because the role of SOCS1 in this critical disease has not been examined *in vivo*, we conducted the present study. We first examined the effects of SOCS1 on BLM-induced pulmonary fibrosis by using SOCS1-haplodeficient mice. Then we explored *SOCS1* mRNA expression in human lung tissues from patients with various types of pathologically proved lung fibrosis. Finally, we examined the effects of *SOCS1* gene transfer on BLM-induced pulmonary fibrosis to evaluate its possible use as therapy.

METHODS

Animals and BLM exposure

This study was approved and conducted in accordance with the guidelines of the animal ethics committee of Hiroshima University. Because SOCS1-deficient mice die within 3 weeks after birth, SOCS1 heterozygotic mice were used to evaluate BLM-induced lung injury.[11] For a separate analysis of the effect of exogenous SOCS1 by means of adenoviral gene transfer, female C57BL/6J mice aged 6 weeks were purchased from Japan Charles River Laboratories (Kanagawa, Japan). All animals were maintained in a specific pathogen-free environment.

For each experiment, age- and weight-matched groups of mice were used. At day 0, after achievement of pentobarbital-induced anesthesia, mice were instilled with BLM (Nippon Kayaku Co, Tokyo, Japan) intratracheally, as previously described.[12] The dose of BLM was 1.5 mg/kg dissolved in sterile PBS (pH 7.4) in all experiments, except the survival-monitoring experiment, in which BLM at a higher dose (3 mg/kg) was used, and mortality was assessed for 20 days. Control mice received PBS alone.

Human lung tissue

Our human study protocol was approved by the Institutional Review Board of Hiroshima University. Between January 2005 and March 2007, lung tissues from 18 Japanese patients (7 female and 11 male patients; age, 43-73 years) with interstitial lung diseases (ILDs) were obtained by means of open lung biopsy performed for diagnostic purposes at Hiroshima University Hospital. From 8 patients with lung cancer, histologically normal lung tissues were obtained from sites distant from the tumors and used as control specimens. Clinical, radiologic, and pathologic analyses revealed a diagnosis of IPF in 10 of the 18 patients, idiopathic nonspecific interstitial pneumonia (NSIP) in 5 patients (2 with cellular and 3 with fibrotic NSIP), and chronic hypersensitivity pneumonitis (CHP) in 3 patients. In 7 of the 10 patients with IPF, 2 of the 5 patients with NSIP, and 3 of the 3 patients with CHP, both upper and lower lung tissues were obtained. The diagnoses of IPF, idiopathic NSIP, and CHP were done by an expert lung pathologist according to guidelines.[1,13] After obtaining informed consent, these lung tissues were stored at −80°C until total RNA extraction (as described below).

Construction and preparation of adenoviral vector expressing SOCS1

Recombinant E1-deleted adenoviral vectors carrying murine *SOCS1* cDNA under the control of a cytomegalovirus promoter (Ad-SOCS1) were generated as previously described.[14] An adenoviral vector expressing the gene for murine β-galactosidase (Ad-LacZ) was used as a control. These viruses were grown in 293 cells and purified by using a commercially available kit (ViraKit AdenoMini Kit; Nacalai Tesque, Inc, Kyoto, Japan). After purification, these viral titers were determined by means of plaque titration in 293 cells and stored at −80°C. A dose of 0.1×10^9 plaque-forming units of vector was diluted in 50 μL of PBS and instilled intranasally 48 hours before the BLM exposure (at day −2), as previously described.[15]

Analysis of bronchoalveolar lavage fluid

Mice were killed by means of cervical dislocation after achievement of anesthesia at days 7 and 14. At the time of death, bronchoalveolar lavage fluid (BALF) was obtained by using a previously described method.[5] The concentrations of murine TNF-α and TGF-β1 in the BALF supernatant were quantified with ELISA kits (R&D Systems, Minneapolis, Minn).

Hydroxyproline assay

Hydroxyproline content of whole mouse lungs was assayed in each group 14 days after BLM administration to evaluate the total amount of lung collagen by using a previously described method.[12]

Purification of RNA and real-time quantitative RT-PCR

Murine left lungs were harvested and stored at −80°C at days 0 (before BLM treatment), 1, 3, and 7. Total RNA was isolated from these murine lungs and human samples by using TRIzol reagent (Invitrogen, Carlsbad, Calif) and purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany). After this, total RNA was reverse transcribed into cDNA, and real-time quantitative RT-PCR was performed with sequence-specific TaqMan primers and probes, the ABI Prism 7500 Fast sequence detector, and SDS analysis software (Applied Biosystems, Foster City, Calif). Mouse samples were analyzed for SOCS1, SOCS3, IL-4, IL-5, IL-10, IL-13, IFN-γ, TGF-β1, TNF-α, monocyte chemoattractant protein 1, eotaxin, and 18S ribosomal protein, and human samples were analyzed for SOCS1 and 18S by using TaqMan Pre-Developed primers and probes (Applied Biosystems), as previously described.[16] The measured mRNA levels were expressed relative to the internal reference 18S mRNA level and further adjusted to the level in the control group at day 0 (which was taken to be one).

Histology

After BALF collection and left lung resection for RNA analysis, the right lung was removed and fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections. The sections were stained with hematoxylin and eosin, or elastica-Masson trichrome. X-gal staining was done in the early (day 3) and late (day 14) phases of BLM treatment with the β-Galactosidase Staining Kit (Mirus Bio Corp, Madison, Wis) to locate and assess gene expression of the adenoviral vector. The paraffin sections were further processed for immunohistochemistry by using rabbit anti-SOCS1 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif), according to a previously described method.[17]

Statistical analysis

Data are shown as means ± SEMs. Differences between groups were analyzed by using the Mann-Whitney *U* test. Differences between paired parameters were tested with the Wilcoxon test. Correlation coefficients for the markers were calculated by using Spearman rank correlation coefficient analysis. The survival function of each group was evaluated with the Kaplan-Meier method, and differences between 2 groups were evaluated with the log-rank test. A *P* value of less than .05 was accepted as statistically significant. All analyses were performed with a statistical software package (SPSS for Windows, version 12.0; SPSS, Inc, Chicago, Ill).

RESULTS

Kinetics of SOCS1 expression in BLM-injured lungs

The time course of *SOCS1* mRNA expression before and after BLM administration is shown in Fig 1, *A*. *SOCS1* mRNA was rapidly induced after BLM treatment from day 1 and maintained to day 3 in wild-type (WT) mice. In $SOCS1^{+/-}$ mice, SOCS1 was also quickly induced after BLM instillation; however, the expression level of SOCS1 was significantly lower in $SOCS1^{+/-}$ mice than in WT mice throughout the time course. The SOCS1 expression was also evaluated by means of immunohistochemistry (Fig 1, *B*). Enhanced expression of SOCS1 was observed in epithelial cells and infiltrated mononuclear and polynuclear cells in mice

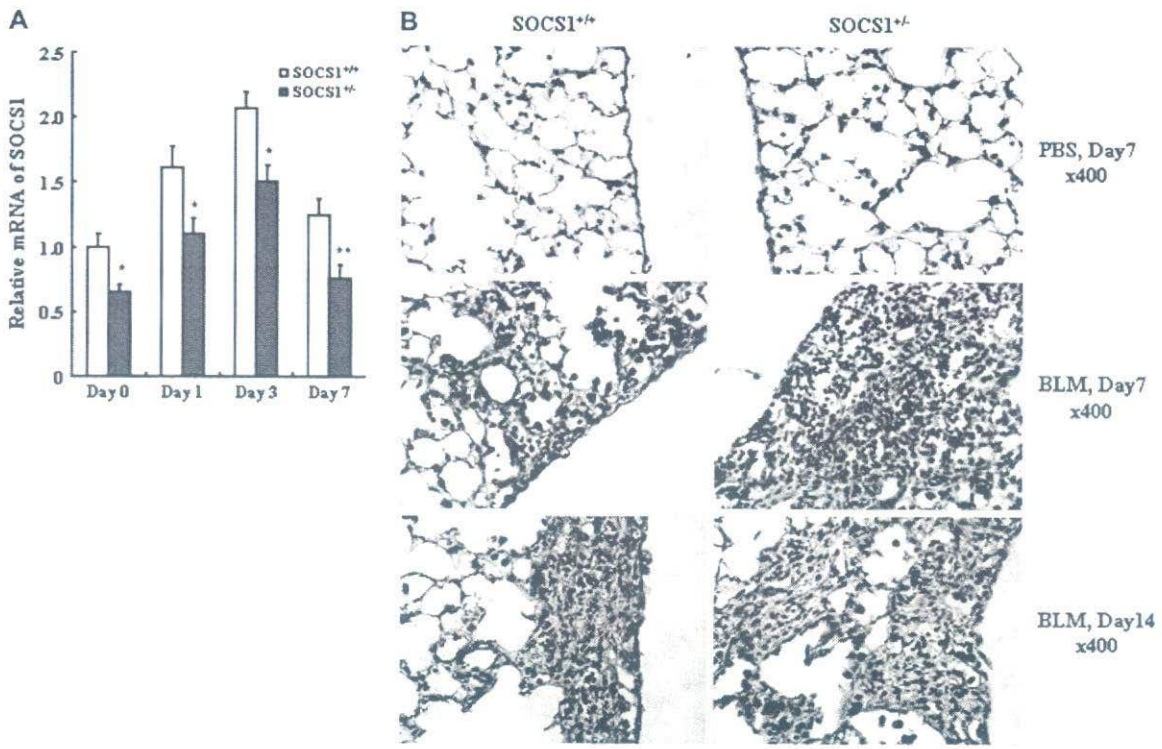

FIG 1. Time course of SOCS1 mRNA **(A)** and protein **(B)** expression in SOCS1$^{+/-}$ and SOCS1$^{+/+}$ mice before and after BLM administration (n = 6 for each group). *P < .05 and **P < .01 between SOCS1$^{+/-}$ and SOCS1$^{+/+}$ mice.

treated with BLM compared with that seen in mice treated with PBS. The SOCS1$^{+/-}$ mice had less expression of SOCS1 protein than WT mice on both days 7 and 14 after treatment with BLM.

Effect of SOCS1 reduction on BLM-induced pulmonary inflammation

The effect of *SOCS1* gene reduction in BLM-induced lung inflammation was evaluated in SOCS1$^{+/-}$ mice. SOCS1$^{+/-}$ mice treated with PBS were similar to SOCS1$^{+/+}$ mice treated with PBS (Fig 2, *A*). However, SOCS1$^{+/-}$ mice treated with BLM had significantly increased numbers of total cells, macrophages, lymphocytes, and eosinophils in BALF obtained on day 7 when compared with SOCS1$^{+/+}$ mice treated in the same manner (Fig 2, *A*). The eosinophilia observed in BALF continued until day 14. In histologic analysis of acute-phase inflammation at day 7, both SOCS1$^{+/-}$ and SOCS1$^{+/+}$ mice treated with PBS showed no apparent inflammation. In contrast, acute inflammatory changes were demonstrated in lung parenchyma of mice after BLM exposure. The intensity of the inflammation observed was much stronger in SOCS1$^{+/-}$ mice than in SOCS1$^{+/+}$ mice (Fig 2, *B*). Markedly increased numbers of lymphocytes and eosinophils were observed in SOCS1$^{+/-}$ mice, which was consistent with the findings in BALF.

Effect of SOCS1 reduction on BLM-induced pulmonary fibrosis

Both lung histopathology and hydroxyproline content of the lungs were analyzed at day 14 to evaluate the effect of SOCS1 on pulmonary fibrosis. After BLM, fibrosis was enhanced in the lungs from SOCS1$^{+/-}$ mice (Fig 3, *A*), and the hydroxyproline content was significantly higher in lungs from SOCS1$^{+/-}$ mice than in lungs from SOCS1$^{+/+}$ mice (Fig 3, *B*), indicating that exacerbation of pulmonary fibrosis was induced by SOCS1 reduction. There were no significant differences in histologic findings and hydroxyproline content between SOCS1$^{+/-}$ and SOCS1$^{+/+}$ mice treated with PBS alone (Fig 3).

Loss of gene regulation in SOCS1$^{+/-}$ mice treated with BLM

Expression of cytokines in the lung was examined by means of real-time quantitative RT-PCR to investigate the underlying mechanisms responsible for markedly enhanced inflammation and fibrosis in SOCS1$^{+/-}$ mice (Fig 4, *A*). Not only T_H2 (*IL4*, *IL5*, and *IL13*) but also T_H1 *(IFNG)* cytokine mRNA levels were significantly increase in SOCS1$^{+/-}$ mice compared with those seen in SOCS1$^{+/+}$ mice. The values for chemokine (eotaxin and monocyte chemoattractant protein 1) and *TNFA* mRNA were also significantly increased in SOCS1$^{+/-}$ mice compared with those in SOCS1$^{+/+}$ mice. SOCS1$^{+/-}$ mice also showed increased expression of SOCS3 compared with WT mice (Fig 4, *B*). On the other hand, the *TGFB1* mRNA level tended to increase in SOCS1$^{+/-}$ mice but did not reach significance during the time examined (data not shown). The protein level of TNF-α was significantly increased at day 7 but not day 14, whereas the enhancement of TGF-β1 level was more prominent at day 14 in the BALF of SOCS1$^{+/-}$ mice (Fig 4, *C*).

Association between SOCS1 expression and severity of pulmonary fibrosis in patients with ILDs

Human lung specimens were investigated to determine whether severity of fibrosis was associated with decreased SOCS1

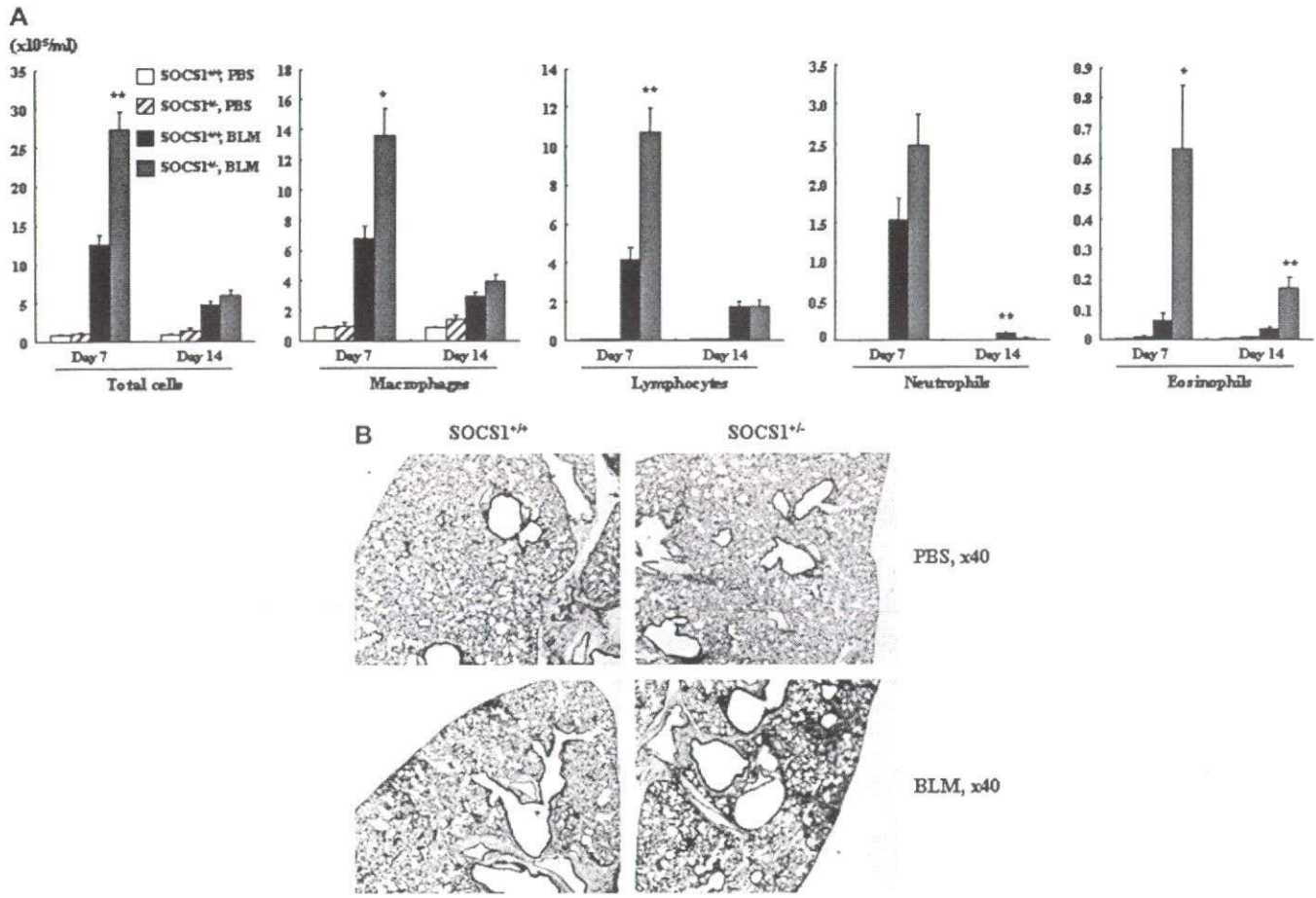

FIG 2. SOCS1 suppresses BLM-induced lung inflammation. **A**, Inflammatory cells in BALF from SOCS1$^{+/-}$ and SOCS1$^{+/+}$ mice (n = 8-12 in each group). *$P < .05$ and **$P < .01$ between SOCS1$^{+/-}$ and SOCS1$^{+/+}$ mice. **B**, Lung histology of SOCS1$^{+/-}$ and SOCS1$^{+/+}$ mice 7 days after PBS or BLM treatment. Lung sections were stained with hematoxylin and eosin.

expression (Fig 5). *SOCS1* mRNA was extracted from histologically assessed lung tissues to evaluate this (see examples in Fig 5, *A*). At first, we found that the *SOCS1* mRNA level was significantly higher in lungs from patients with IPF than in control lungs ($P < .05$, data not shown). Next the relationship between *SOCS1* mRNA expression level and clinical data (including age, sex, time after onset of first symptoms, blood gas analysis data, and lung function data) was determined. As shown in Fig 5, *B*, the *SOCS1* mRNA expression level was negatively correlated with the duration of the disease ($P = .00015$). Furthermore, *SOCS1* mRNA levels were significantly less in lungs from patients with IPF than in lungs from patients without IPF (NSIP and CHP; $P = .003$; Fig 5, *C*). We also found that the level of *SOCS1* mRNA expression in lung tissue with more severe fibrotic lesions (lower lobe) was significantly lower than in lung tissue with less severe fibrotic lesions (upper lobe) in the same patients ($P = .015$; Fig 5, *D*).

Exogenous SOCS1 delivered by means of adenoviral gene transfer ameliorates BLM-induced pulmonary inflammation, pulmonary fibrosis, and mortality

Next the effect of exogenous SOCS1 on BLM-induced lung injury was examined (Fig 6). At first, localization of adenovirus-mediated gene expression was confirmed in the nucleus of airway epithelial cells and alveolar epithelial cells, which was demonstrated by means of X-gal staining throughout the time course (from day 3 to day 14; data not shown). Real-time quantitative RT-PCR analysis revealed significantly higher levels of SOCS1 in *SOCS1* gene-transfected mice than in control mice over the entire time course ($P < .01$ for day 0 to day 3 and $P < .05$ for day 7, data not shown).

As shown in Fig 6, *A*, treatment with the *SOCS1* gene protected mice from BLM-induced death and improved survival of mice exposed to high-dose BLM (3 mg/kg) compared with control mice (83.3% of mice infected with Ad-SOCS1 vs 41.7% of mice infected with Ad-LacZ, $P = .040$). In the pulmonary fibrosis model induced by usual-dose BLM (1.5 mg/kg), transfer of the *SOCS1* gene significantly inhibited acute lymphocytic inflammation in BALF only at day 7 (Fig 6, *B*). Reduced lung hydroxyproline content (Fig 6, *C*) also indicated pronounced inhibition of pulmonary fibrosis. Expression of T_H2 cytokines (IL-4 and IL-13) was suppressed in the Ad-SOCS1 group (compared with the control group; $P < .05$ for day 1 to day 3 in IL-4 and $P < .01$ for day 1 in IL-13), but expression of the T_H1 cytokine (IFN-γ) was not affected (Fig 6, *D*). Exogenous SOCS1 also significantly inhibited both profibrotic *TNFA* mRNA at days 1 and 3 (Fig 6, *D*) and *TGFB1* mRNA at day 3

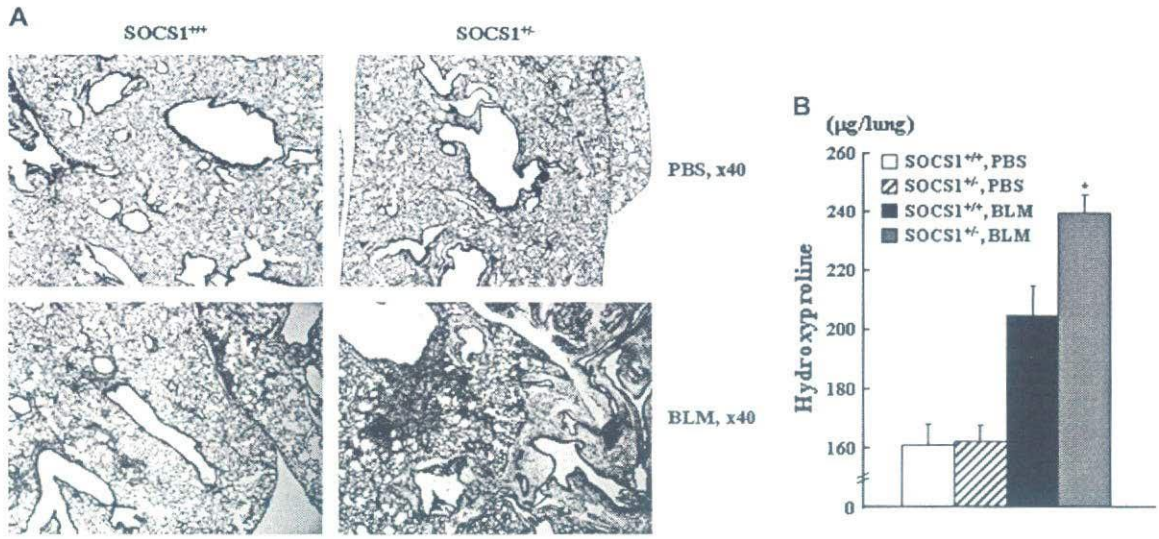

FIG 3. SOCS1 regulates BLM-induced pulmonary fibrosis. A, Histologic sections stained with elastica-Masson trichrome were examined for pulmonary fibrosis. B, Quantification of hydroxyproline content of whole lungs of SOCS1$^{+/-}$ and SOCS1$^{+/+}$ mice (n = 8 per group) was used to assess pulmonary fibrosis. *$P < .05$ between SOCS1$^{+/-}$ and SOCS1$^{+/+}$ mice.

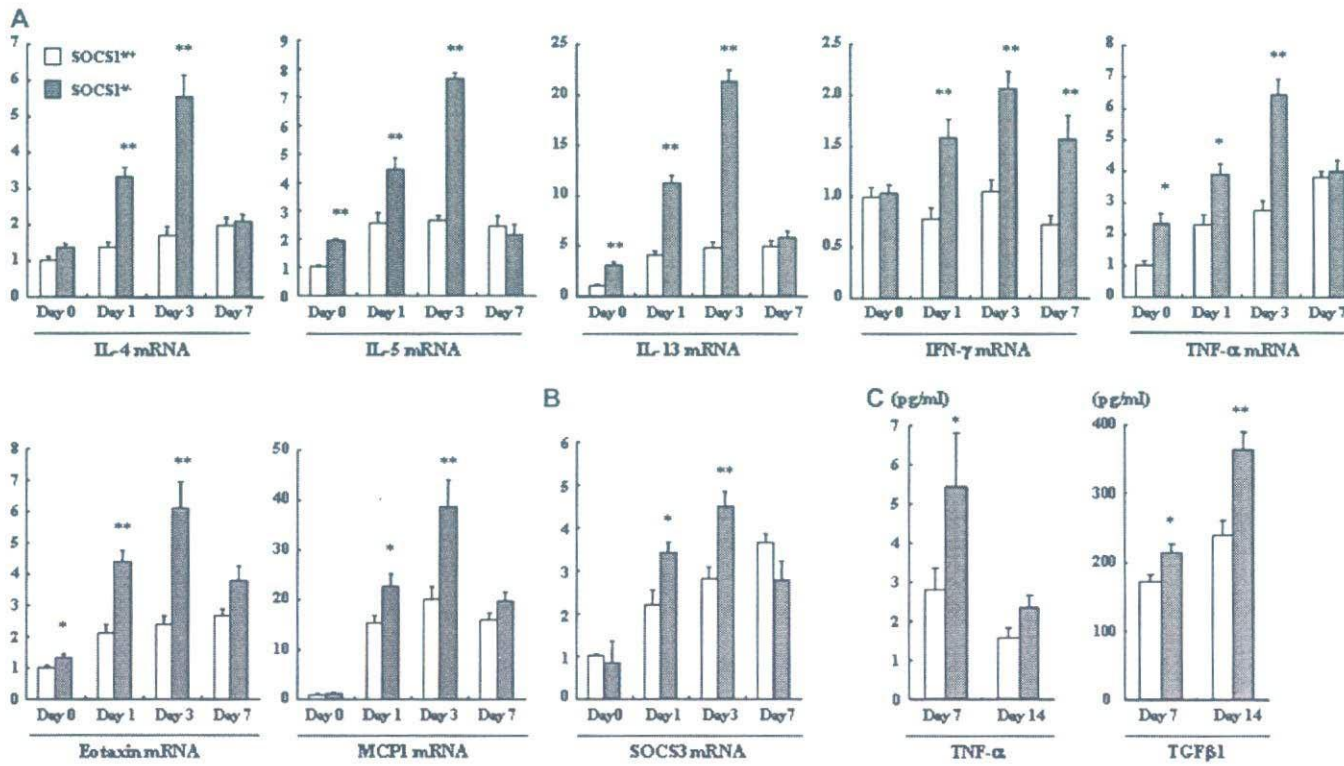

FIG 4. SOCS1 deficiency causes enhanced gene and protein expression during BLM-induced lung injury. Kinetics of cytokine, chemokine (A), and *SOCS3* (B) mRNA expression in SOCS1$^{+/-}$ and SOCS1$^{+/+}$ mice (n = 6 in each group). C, Concentrations of TNF-α and TGF-β1 in BALF. *$P < .05$ and **$P < .01$ between SOCS1$^{+/-}$ and SOCS1$^{+/+}$ mice. *MCP1*, Monocyte chemoattractant protein 1.

(data not shown). In this case expression of *SOCS3* mRNA was not different between the Ad-SOCS1 and Ad-LacZ groups of mice (Fig 6, *E*). Furthermore, the Ad-SOCS1 group showed decreased protein levels of both TNF-α and TGF-β1 in BALF compared with Ad-LacZ group. The inhibition of TNF-α was more prominent in the earlier phase (day 7), whereas it was more prominent in the later phase (day 14) in the case of TGF-β1 (Fig 6, *F*).

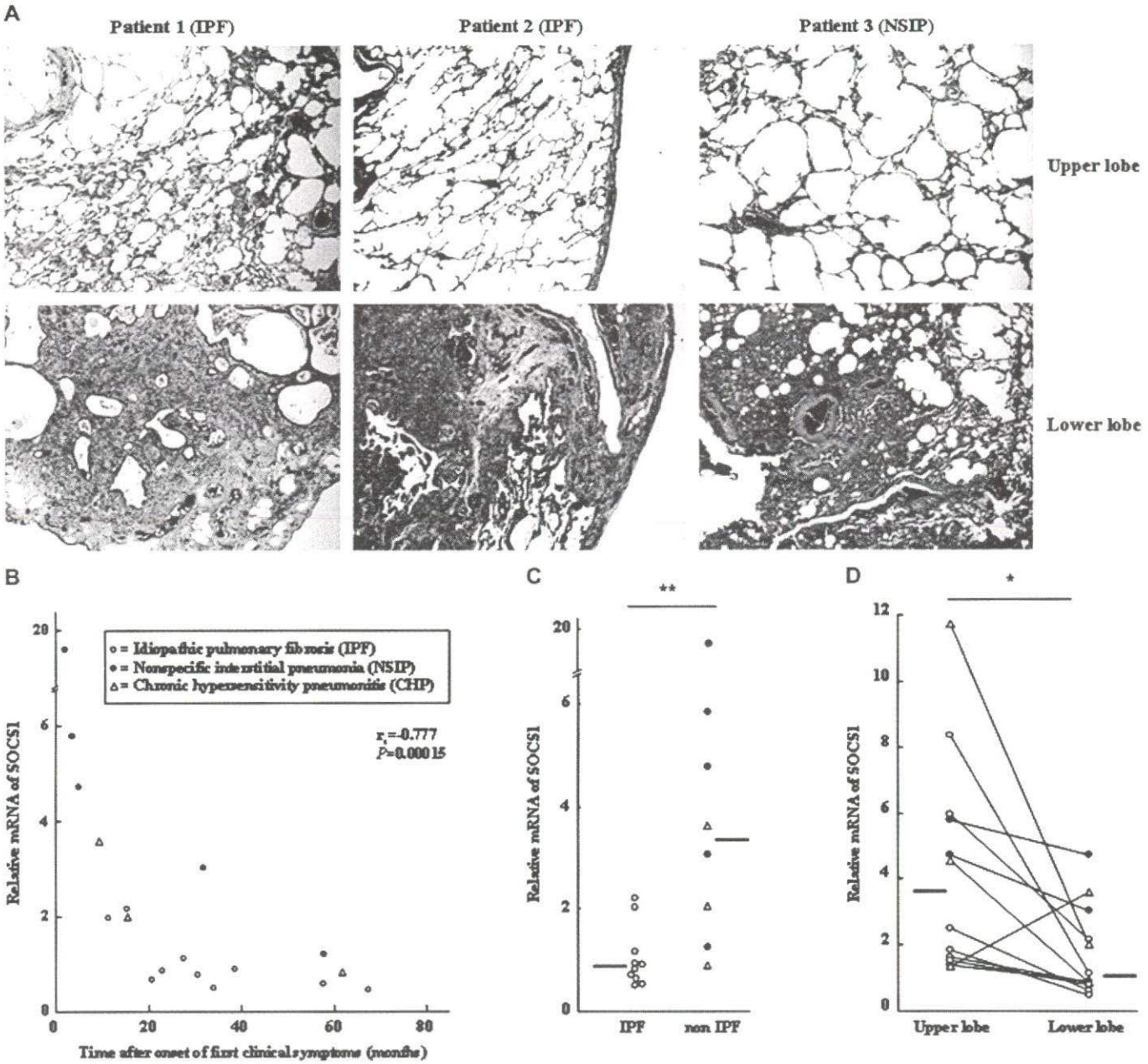

FIG 5. Relationship between SOCS1 expression and severity of fibrosis in patients with ILDs. **A**, Examples of histologic assessment. **B**, SOCS1 expression and duration of ILDs. **C**, *SOCS1* mRNA expression in the lower lobes of lungs from patients with ILDs. **D**, Comparison of SOCS1 expression between mild and severe fibrotic areas in each patient. $*P < .05$ and $**P < .01$ between 2 groups.

DISCUSSION

The current study clearly demonstrates that SOCS1 plays a role in BLM-induced pulmonary inflammation and fibrosis *in vivo*. SOCS1 downregulation in SOCS1$^{+/-}$ mice resulted in the exacerbation of pulmonary inflammation and fibrosis, in conjunction with enhancement of profibrotic cytokine and chemokine production. Analysis of lung specimens from patients with ILDs found an inverse correlation between SOCS1 expression and duration of the disease. Lung samples from patients with IPF expressed significantly less *SOCS1* mRNA than did samples from patients without IPF, such as patients with NSIP and CHP. *SOCS1* mRNA expression was significantly less in histologically proved severe fibrotic lesions than in histologically proved mild fibrotic lesions. Furthermore, we found that exogenous SOCS1 induced regression of pulmonary fibrosis, decreased lymphocytic inflammation, and protected against mortality caused by high-dose BLM. Our previous study demonstrated that the enhanced collagen production by lung fibroblasts from patients with IPF was causally related to diminished expression of SOCS1.[10] The present study was consistent with this observation and further clarified the novel role of SOCS1 as a suppressor of pulmonary fibrosis *in vivo*.

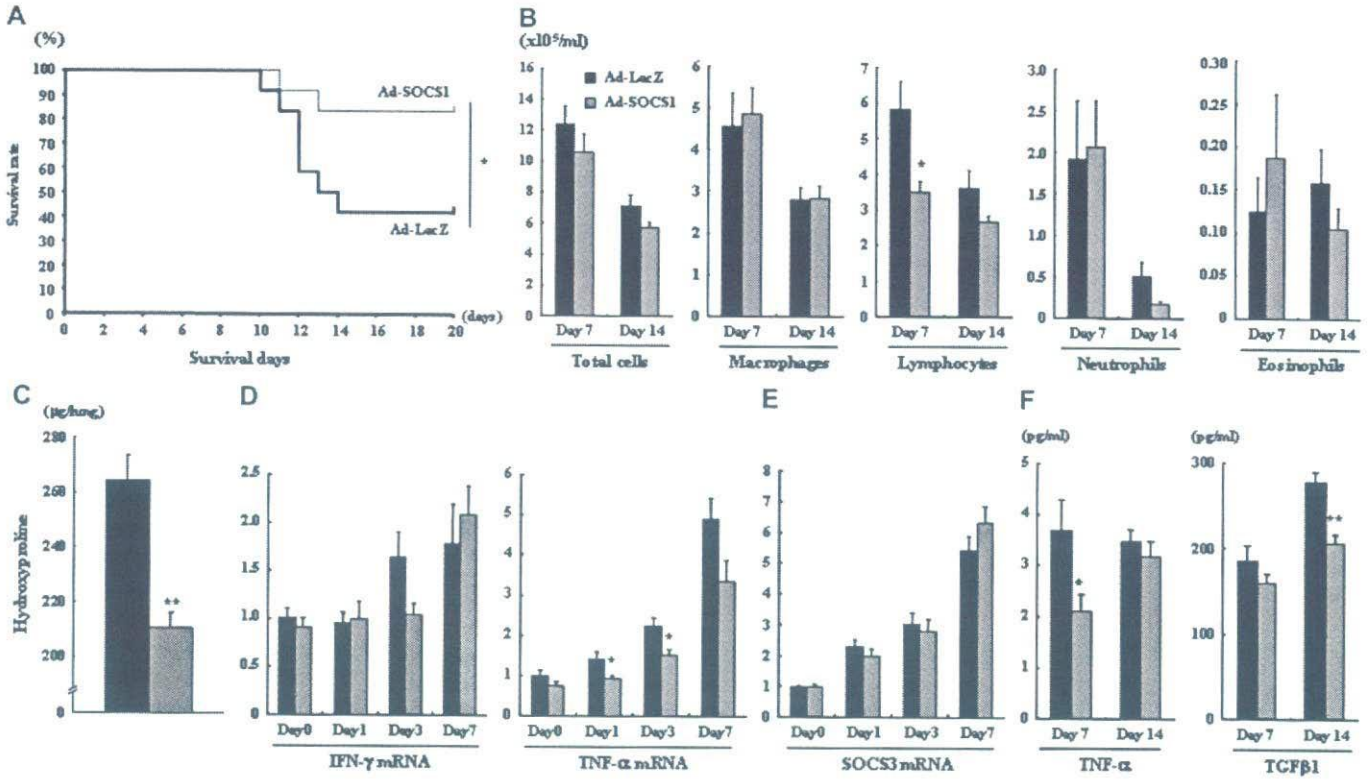

FIG 6. Inhibition of BLM-induced lung injury by adenovirus-mediated exogenous SOCS1 (n = 6-12 per group). Effects of exogenous SOCS1 on survival (**A**), inflammatory cells in BALF (**B**), and hydroxyproline assay (**C**). Time course of cytokine (**D**) and *SOCS3* (**E**) mRNA expression. **F**, Concentration of TNF-α and TGF-β1 in BALF. $*P < .05$ and $**P < .01$ between the Ad-SOCS1 and Ad-LacZ groups.

To date, there is little evidence linking SOCS proteins with pulmonary diseases[18-20] and no previous report showing the role of SOCS1 in pulmonary fibrosis. A recent article suggested that SOCS1 is a suppressor of liver fibrosis.[21] In this study enhanced dimethylnitrosamine-induced liver inflammation and fibrosis accompanied by increased TGF-β production were observed in $SOCS1^{+/-}$ mice compared with that seen in $SOCS1^{+/+}$ mice. Our current study similarly demonstrated enhanced BLM-induced lung inflammation and fibrosis in $SOCS1^{+/-}$ mice compared with that seen in $SOCS1^{+/+}$ mice. Like the enhancement in liver fibrosis, the enhancement in lung fibrosis in $SOCS1^{+/-}$ mice depended on TGF-β production.

We showed that SOCS1 might be used for pulmonary fibrosis treatment. Exogenous SOCS1 transferred by an adenovirus vector induced beneficial effects in a BLM-induced pulmonary fibrosis model. These benefits were due to the multisuppressive effects of SOCS1. Among the molecules affected by SOCS1, we believe TNF-α might be the most important for several reasons.

First, expression of TNF-α was decreased in the lungs after introduction of exogenous SOCS1. TNF-α has pleiotropic and differential effects, including triggering of lymphocytic inflammation and induction of other cytokines (eg, T_H2 cytokines), cell proliferation, differentiation, and apoptosis, resulting in enhancement of pulmonary fibrosis.[3,22] A number of studies have demonstrated the profibrotic significance of TNF-α in pulmonary fibrosis, not only in animal BLM models but also in human subjects with IPF.[3,23-26]

Second, thus far, SOCS1 (but not other members of the SOCS family) is reported to regulate signals of TNF-α.[7,9] In particular, a recent study suggested that SOCS1 is a suppressor of TNF-α–induced apoptosis.[27] Therefore exogenous SOCS1 not only can decrease production of TNF-α but also can inhibit the function of TNF-α.

Third, TNF-α expression in lungs injured by BLM injection was localized to bronchial epithelial cells, in particular apoptotic epithelial cells.[24] Thus airway epithelial cells (the target of adenovirus-mediated gene upregulation) are the main source of TNF-α in BLM-induced lung injury. In fact, increase of TNF-α gene and protein expression is observed in cells from $SOCS1^{-/-}$ mice in response to stimuli.[7]

Finally, TNF-α is necessary for TGF-β1 induction in this model.[24]

It should be noted that $SOCS1^{+/-}$ mice treated with BLM were affected by eosinophilic inflammation, whereas exogenous SOCS1 did not affect eosinophilic inflammation. Because the location of affected SOCS1 expression was different between $SOCS1^{+/-}$ mice and the Ad-SOCS1 group of mice, these mice should not be a mirror image. Transfer of Ad-SOCS1 was limited in airway epithelial cells, whereas expression of SOCS1 was systemically decreased in $SOCS1^{+/-}$ mice. As a consequence, $SOCS1^{+/-}$ mice showed enhanced expression of SOCS3 compared with WT mice, whereas expression of *SOCS3* mRNA was not changed in mice with Ad-SOCS1. Because enhanced SOCS3 expression in T or dendritic cells was reported to be associated with a T_H2-dominant response,[19,28] induction of SOCS3 might be an explanation for the enhanced eosinophilic inflammation observed in $SOCS1^{+/-}$ mice.

In the steady state SOCS1 expression remains at very low levels. However, many cytokines and other stimuli immediately

induce SOCS1. One recent hypothesis for the pathogenesis of IPF is repeated lung injury by an unidentified stimulus and pulmonary fibrosis caused by aberrant wound healing after the repeated lung injury.[1] From this point of view, our results suggest that an unidentified stimulus induced SOCS1, which was upregulated in lung tissues of patients with IPF relative to that of control subjects. Our previous data suggested that spontaneous expression of SOCS1 is reduced in fibroblasts from lung tissues from patients with IPF compared with those from control lung tissues.[10] In addition, we discovered that SOCS1 expression in lung tissues from patients with ILD was negatively correlated with the duration of the disease. This fact also suggested a relationship between SOCS1 and the degree of fibrosis because the fibrosis gradually gets worse in the usual clinical course of IPF. One possible mechanism is that continuous stimulation might exhaust the supply of SOCS1 and insufficient SOCS1 might lead to more severe fibrosis. In accordance with the above speculation, diminished SOCS1 expression was observed in patients with IPF compared with that seen in patients without IPF (NSIP and CHP) and in severe fibrotic lesions (lower lobe) relative to less fibrotic lesions (upper lobe).

In conclusion, our results strongly suggest that a decrease in SOCS1 expression is involved in accelerated pulmonary fibrosis in both animals and human subjects. This notion raises the possibility that SOCS1 could be a novel target for treating lung fibrosis.

We thank Dr Ayumu Nakashima for his excellent technical assistance.

Clinical implications: Our results strongly suggest that a decrease in SOCS1 expression is involved in accelerated pulmonary fibrosis in both animals and human subjects. SOCS1 could be a novel target for treating lung fibrosis.

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Suplatast Tosilate Prevents Bleomycin-Induced Pulmonary Fibrosis in Mice

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ABSTRACT

Increasing evidence suggests that the development of pulmonary fibrosis is a T helper (Th) 2-mediated process. Suplatast tosilate is a Th2 cytokine inhibitor that is widely used as an asthma controller in Japan. Therefore, we hypothesized that suplatast tosilate might have an inhibitory effect on the development of pulmonary fibrosis. To investigate this effect, suplatast tosilate was administered to mice after the intratracheal instillation of bleomycin (BLM). The effect of suplatast tosilate was studied by analysis of bronchoalveolar lavage (BAL) fluid and a hydroxyproline assay. We found that the treatment of mice with suplatast tosilate significantly reduced the degree of pulmonary fibrosis. Because a significantly elevated Th2 response was not detected in the C57BL/6 mice after BLM administration, the effect of suplatast tosilate on Th2 cytokines could not be demonstrated. Interestingly, however, the up-regulation of the monocyte chemoattractant protein (MCP)-1 levels in the BAL fluid was found to be suppressed. Following these results, we also demonstrated that suplatast tosilate effectively inhibited the production of MCP-1 in alveolar macrophages (AMs). These findings suggest that suplatast tosilate has both anti-inflammatory and antifibrotic effects, which were associated with a suppressed MCP-1 expression in AMs. Thus, suplatast tosilate, which is already widely used in Japan, may warrant further consideration as a potentially useful treatment for pulmonary fibrosis.

Idiopathic pulmonary fibrosis (IPF) is defined as a specific form of chronic fibrotic interstitial pneumonia associated with the histopathological appearance of usual interstitial pneumonia. The median survival of patients with IPF is reported to be 3 to 4 years from the onset of respiratory symptoms (American Thoracic Society, 2000). Despite such a poor prognosis, the etiology of IPF remains unknown, and no effective therapeutic strategy has yet been established. The effects of current immunosuppressive therapy with corticosteroids and cytotoxic agents are limited, and the adverse effects cannot be ignored. Therefore, the establishment of an alternative therapeutic strategy is urgently needed.

Because the overall cytokine pattern in biopsies and alveolar macrophages from patients with IPF appears to be more Th2-type (i.e., IL-4, IL-5, and IL-13) than Th1-type (i.e., IL-12 and IFN-γ) (Wallace et al., 1995; Furuie et al., 1997), a biased Th2 cytokine profile in the lungs is regarded as one of the causes of IPF; thus, a therapeutic strategy to correct this bias seems to be a promising approach. To the best of our knowledge, IFN-γ, a major Th1 cytokine, is the only agent used for IPF patients in clinical studies as a therapeutic strategy-targeted inhibition of Th2 cytokines. The rationale for its use was also based on its properties as an inhibitor of fibroblast proliferation, collagen synthesis, and deposition. A recent meta-analysis study showed IFN-γ therapy to be associated with a reduced mortality (Bajwa et al., 2005); however, a randomized controlled trial involving 330 patients failed to show any clear benefit from IFN-γ therapy in comparison with a placebo (Raghu et al., 2004; King et al., 2005).

Seeking other means to modulate Th2 cytokines in the lungs stimulated interest in suplatast tosilate (IPD-1151T, Fig. 1), which is widely used as an asthma control drug in Japan because it has been shown to have an inhibitory effect

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ABBREVIATIONS: IPF, idiopathic pulmonary fibrosis; Th, T helper; IL, interleukin; IFN, interferon; IPD-1151T, (6)-[2-[4-(3-ethoxy-2-hydroxypropoxy) phenylcarbamoyl] ethyl] dimethylsulfonium *p*-toluenesulfonate; BAL, bronchoalveolar lavage; TGF, transforming growth factor; MCP, monocyte chemoattractant protein; BLM, bleomycin; PBS, phosphate-buffered saline; ST, suplatast tosilate; DW, distilled water; H&E, hematoxylin and eosin; ELISA, enzyme-linked immunosorbent assay; AM, alveolar macrophage; LPS, lipopolysaccharide.