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Overexpression of the Transcription Factor GATA-3 Enhances the Development of Pulmonary Fibrosis

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Recent studies have demonstrated that Th2 cytokines, such as interleukin-4 and interleukin-13, enhance fibrotic processes by activating fibroblast proliferation and collagen production, whereas interferon- γ , a Th1 cytokine, inhibits these processes. Th1 and Th2 cells both differentiate from common T precursor cells, with transcription factor GATA-3 a key regulator of Th2 differentiation. In the present study, therefore, we examined the effects of GATA-3 overexpression on the development of pulmonary fibrosis in a mouse model. Wild-type C57BL/6 mice and GATA-3-overexpressing (*GATA-3-tg*) mice of the same background were intratracheally treated with bleomycin. The survival rate after bleomycin was significantly decreased in *GATA-3-tg* mice compared with wild-type mice. The degree of pulmonary fibrosis was much greater in *GATA-3-tg* mice than in wild-type mice 28 days after bleomycin treatment. Lung interferon- γ concentration was significantly decreased in *GATA-3-tg* mice compared with wild-type mice by 7 days after either saline or bleomycin treatment. The concentration of transforming growth factor- β , a fibrogenic cytokine, was significantly higher in *GATA-3-tg* mice than in wild-type mice. Exogenous administration of interferon- γ to *GATA-3-tg* mice improved the degree of pulmonary fibrosis and thus increased survival. These results indicate that overexpression of GATA-3 enhances the development of pulmonary fibrosis, possibly by reducing interferon- γ levels in the lung. (*Am J Pathol* 2006, 169:96–104; DOI: 10.2353/ajpath.2006.051160)

understood, initial immune and inflammatory responses to the repeated stimuli lead to tissue injury and progressive fibrosis.¹ Histological evaluation of lung tissue from patients with pulmonary fibrosis shows various degrees of inflammation in which many types of inflammatory cells, including macrophages, neutrophils, and lymphocytes, have infiltrated.

Bleomycin is an anti-tumor drug often used as an inducing agent in models of pulmonary fibrosis. Intratracheal administration of bleomycin in rodents induces progressive lung inflammation followed by varying degrees of fibrosis that models human pulmonary fibrosis such as idiopathic pulmonary fibrosis.^{2–4} It is interesting that the accumulation of lymphocytes in the lung is observed 5 to 7 days after bleomycin administration, preceding the development of pulmonary fibrosis.^{5,6}

Among lymphocytes, T cells are the most likely to participate in the development of bleomycin-induced pulmonary fibrosis. Depletion of T cells by treatment with anti-CD4 and anti-CD8 antibodies inhibits the fibrotic response to bleomycin.⁷ Bleomycin-induced pulmonary inflammation and fibrosis are not observed in athymic nude mice.⁸ The mechanisms by which T cells contribute to the fibrotic response have not been clarified but likely involve the production of T-cell-derived cytokines.

Researchers have demonstrated that the specific cytokine phenotype may provide fundamental mechanisms for regulation of the fibrotic process. For example, interleukin (IL)-4 and IL-13 enhance the fibrotic process by augmenting fibroblast proliferation and collagen production.^{9–11} Conversely, interferon- γ (IFN- γ) induces anti-fibrotic effects directly by suppressing fibroblast activity such as proliferation and collagen production^{11–13} or indirectly by attenuating the effects of IL-4 and IL-13.¹⁴ Interestingly, these cytokines are prototypic cytokines participating in type 1 (Th1) and type 2 (Th2) immune responses. These findings therefore suggest that Th1 differentiation (and expression of IFN- γ) attenuates the

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Pulmonary fibrosis is an end-stage disorder with a poor prognosis. Although the fibrotic process is not yet fully

development of pulmonary fibrosis whereas Th2 differentiation (and expression of IL-4 and IL-13) aggravates it.

Th1 and Th2 cells are differentiated from common T-precursor cells.^{15,16} The differentiation requires the activity of distinct transcription factors. Among a variety of key molecules governing Th1/Th2 differentiation, GATA-3 has been implicated in Th2 commitment.^{17–20} Under physiological conditions, GATA-3 is selectively expressed in Th2 but not Th1 cells.^{17,18,21} Transgenic and retroviral expression of GATA-3 induces a Th2 cytokine profile in Th1 cells,^{17,18} whereas dominant-negative GATA-3 down-regulates this profile in Th2 clones.²¹ We recently established GATA-3-overexpressing transgenic mice.²² In the present study, using these mice, we evaluated the role of GATA-3 overexpression in the development of bleomycin-induced pulmonary fibrosis.

Materials and Methods

Animals

Wild-type C57BL/6 mice were obtained from Charles River Laboratories (Kanagawa, Japan). Heterozygous GATA-3-overexpressing transgenic (*GATA-3-tg*) mice generated in our laboratory²² were backcrossed with C57BL/6 mice for eight generations. All mice used in this study were 6 to 8 weeks of age and were maintained in our animal facilities under specific pathogen-free conditions. All animal studies were approved by the institutional review board at our facility. Mice were administered bleomycin (5 mg/kg; Calbiochem, San Diego, CA) or saline intratracheally.

Histopathological Assessment

The lungs were removed 28 days after bleomycin or saline administration. After fixation, the lungs were embedded in paraffin. The sections were then stained with Masson's trichrome stain. The grade of pulmonary fibrosis was scored on a scale of 0 to 8 using a previously described scoring method.²³ After the examination of 30 randomly chosen regions in each sample at a magnification of $\times 100$, the mean score of all of the fields was taken as the fibrosis score in each sample.

Assessment of Collagen Synthesis

Collagen synthesis was assessed using a hydroxyproline assay. The mice were anesthetized, and the lungs were removed 28 days after bleomycin or saline administration. Hydroxyproline content was measured as reported previously.²⁴

Bronchoalveolar Lavage

One and seven days after bleomycin or saline administration, the lungs were lavaged with six sequential aliquots of 1 ml of phosphate-buffered saline (PBS). The remaining pooled bronchoalveolar lavage was centri-

fuged and resuspended in PBS. Cells were counted using a hemocytometer, and a differential cell count was performed by standard light microscopic techniques based on staining with Diff-Quik (American Scientific Products, McGraw Park, IL).

Flow Cytometry

Seven days after bleomycin or saline administration, the lungs were removed, minced, and incubated with RPMI 1640 containing 10% fetal bovine serum and 75 U/ml collagenase (type 1; Sigma Chemical Co., St. Louis, MO) at 37°C for 90 minutes. The cells were then filtered through 20- μ m nylon mesh. The cell suspensions were stained with anti-CD4 (BD PharMingen, San Diego, CA), anti-CXCR3 (R&D Systems, Minneapolis, MN), anti-CCR3 (R&D Systems), and anti-CD25 antibodies (BD PharMingen), respectively. After staining, the cells were analyzed by flow cytometry using the FACSCaliber with CellQuest software (BD Biosciences, San Jose, CA). Intracellular production of IFN- γ was also determined by flow cytometric intracellular cytokine analysis as previously described.²⁵

Measurement of Cytokines

The concentrations of IFN- γ , IL-4, IL-5, and IL-13 in the lung homogenates were determined by enzyme-linked immunosorbent assay according to the manufacturer's instructions (Endogen, Cambridge, MA). A concentration of the active form of transforming growth factor (TGF)- β was also determined by enzyme-linked immunosorbent assay (R&D Systems).

Treatment with IFN- γ

For continuous exposure of IFN- γ , Alzet osmotic pumps (model no. 1002; Durect, Cupertino, CA) with a 14-day pumping capacity and an infusion rate of 0.25 μ l/hour were used. Pumps were filled with 1.7×10^8 U/ml mouse recombinant IFN- γ (BD PharMingen) diluted in PBS. One day before bleomycin administration, pumps were implanted into the intrascapular subcutaneous space. In control mice, PBS was continuously administered using the same pumps.

Statistics

Data are expressed as the mean $\pm$ SEM. Comparisons of data among the experimental groups were performed using analysis of variance and Scheffé's test. The survival curves were analyzed using the log-rank test. Values of $P < 0.05$ were considered to be statistically significant.

Results

Assessment of Pulmonary Fibrosis

We first evaluated the survival of mice after bleomycin administration. Thirty-five percent of the wild-type C57BL/6

Figure 1. The survival rate is decreased in GATA-3-overexpressing mice after bleomycin treatment. The survival rates of wild-type mice (WT, **circles**) or GATA-3-overexpressing mice (GATA-3, **squares**) after intratracheal administration of 5 mg/kg bleomycin (bleo, **filled**) or saline (saline, **open**). $n = 20$ in each group. *Significant difference ($P < 0.05$) between the WT-bleo and GATA-3-bleo groups.

mice and sixty-five percent of the GATA-3-*tg* mice died by 28 days after bleomycin administration (Figure 1). The survival rate after bleomycin treatment was significantly decreased in GATA-3-*tg* mice compared with wild-type mice. No mice died in the saline-administered control group of either genotype (Figure 1).

We next assessed the development of bleomycin-induced pulmonary fibrosis in both wild-type mice and GATA-3-*tg* mice. Figure 2 shows the lung histology 28 days after bleomycin or saline administration. Masson's trichrome stain revealed mild thickening of alveolar septa and collagen deposition 28 days after bleomycin administration in the lungs of wild-type mice (Figure 2A, WT-bleo). The degree of pulmonary fibrosis was much greater in GATA-3-*tg* mice than in wild-type mice. Dense fibrosis with prominent collagen deposition was observed 28 days after bleomycin administration in the lung of GATA-3-*tg* mice (Figure 2A, GATA-3-bleo). No abnormal alveolar architecture was observed in the lungs of the saline-administered control group in either wild-type mice or GATA-3-*tg* mice (Figure 2A, WT-saline and GATA-3-saline).

We then assessed the degree of pulmonary fibrosis using a scoring method. In mice of both genotypes, the scores of fibrotic lesions were significantly increased 28 days after bleomycin administration, compared with saline-administered controls (Figure 2B). However, the score was significantly higher in GATA-3-*tg* mice than in wild-type mice at that time (Figure 2B).

We further assessed the degree of pulmonary fibrosis by measuring the lung hydroxyproline content, which we found to be significantly increased 28 days after bleomycin administration in both genotypes (Figure 2C). However, the concentration was significantly higher in GATA-3-*tg* mice than in wild-type mice at that time (Figure 2C). These results clearly indicate that the degree of bleomycin-induced pulmonary fibrosis was much greater in mice overexpressing the GATA-3 gene.

Assessment of Pulmonary Inflammation after Bleomycin Treatment

To evaluate the inflammatory phenotype in the early phase of bleomycin treatment, the lungs were lavaged 1

Figure 2. Bleomycin-induced pulmonary fibrosis is aggravated in GATA-3-overexpressing mice. **A:** Photomicrograph of the lung tissues from wild-type mice (WT) and GATA-3-overexpressing mice (GATA-3) 28 days after the intratracheal administration of 5 mg/kg bleomycin (bleo) or saline. Masson's trichrome staining. The score of fibrosis (**B**) and lung hydroxyproline contents (**C**) of wild-type mice (WT) and GATA-3-overexpressing mice (GATA-3) 28 days after intratracheal administration of 5 mg/kg bleomycin (bleo) or saline. $n = 6$ in each group. *Significant difference ($P < 0.05$) between the WT-bleo and GATA-3-bleo groups. Scale bar = 100 μ m.

day and 7 days after bleomycin administration in both wild-type mice and GATA-3-*tg* mice. The number of lavageable macrophages increased significantly 7 days after bleomycin administration in mice of both genotypes, compared with mice before bleomycin administration (Figure 3A). Similarly, the number of lymphocytes was increased significantly 7 days after bleomycin administration in the lungs of mice of both genotypes (Figure 3B). The number of neutrophils was significantly elevated 1 day after bleomycin administration in the lungs of mice of both genotypes (Figure 3C). However, these numbers were not different between wild-type mice and GATA-3-*tg* mice at any time points (Figure 3, A–C). These results indicate that the degree of initial inflammation induced by bleomycin was not different between wild-type mice and GATA-3-*tg* mice.

Assessment of Lung Cytokine Production during the Development of Pulmonary Fibrosis

GATA-3 is a transcription factor that regulates Th2 differentiation. We therefore assessed Th1/Th2 cytokine bal-

Figure 3. The degree of initial pulmonary inflammation induced by bleomycin is not different between wild-type mice and GATA-3-overexpressing mice. The number of macrophages (A), lymphocytes (B), and neutrophils (C) in bronchoalveolar lavage fluid of wild-type mice (WT) and GATA-3-overexpressing mice (GATA-3) before and 1 day and 7 days after administration of 5 mg/kg bleomycin. $n = 6$ in each group. *Significant difference ($P < 0.05$) compared with mice before bleomycin administration.

ance in the lungs of both wild-type mice and GATA-3-*tg* mice. As indicated above, lymphocytes are predominantly infiltrated into the lung 7 days after bleomycin administration in this model. Accordingly, we determined the concentration of IFN- γ , IL-4, IL-5, and IL-13 in lung homogenates at that time point. The concentration of IFN- γ in the lung homogenates was significantly lower in GATA-3-*tg* mice than in wild-type mice under both saline- and bleomycin-treated conditions (Figure 4A). However, the concentrations of IL-4, IL-5, and IL-13 in the lung homogenates were not significantly different between mouse genotypes or by treatment with bleomycin (Figure 4, B–D).

Because GATA-3 is a transcription factor mainly expressed in T cells, we next assessed the production of IFN- γ in CD4 $^{+}$ T cells obtained from the lungs of wild-type mice and GATA-3-*tg* mice 7 days after administration of bleomycin or saline. In the saline-administered control group, less than 10% of the CD4 $^{+}$ T cells were positive for IFN- γ , and there was no difference in IFN- γ production between wild-type mice and GATA-3-*tg* mice (Figure 5A). The proportion of IFN- γ -producing CD4 $^{+}$ T cells increased significantly in wild-type mice after bleomycin administration (Figure 5A). However, the propor-

tion of IFN- γ -producing CD4 $^{+}$ T cells was still low in GATA-3-*tg* mice after bleomycin administration (Figure 5A). These results indicate that the production of IFN- γ by stimulation with bleomycin was suppressed in CD4 $^{+}$ T cells of GATA-3-*tg* mice.

We further assessed the production of IFN- γ in the subpopulation of CD4 $^{+}$ T cells in the lungs of mice of both genotypes. The proportion of CD4 $^{+}$ CXCR3 $^{+}$ cells, which represents Th1 cells, in CD4 $^{+}$ T cells was significantly higher in the lungs of wild-type mice than in those of GATA-3-*tg* mice at 7 days after bleomycin administration (Figure 5B, top panels). Moreover, the proportion of IFN- γ -positive cells in CD4 $^{+}$ CXCR3 $^{+}$ cells was higher in wild-type mice than in GATA-3-*tg* mice (Figure 5C, top panels). The proportion of CD4 $^{+}$ CCR3 $^{+}$ cells, which represent Th2 cells, in CD4 $^{+}$ T cells was less than 5% in the lungs of both genotypes (Figure 5B, middle panels). The proportion of IFN- γ -positive cells in CD4 $^{+}$ CCR3 $^{+}$ cells was not different between wild-type mice and GATA-3-*tg* mice (Figure 5C, middle panels). Although the proportion of CD4 $^{+}$ CD25 $^{+}$ regulatory T cells in CD4 $^{+}$ T cells was not different between wild-type mice and GATA-3-*tg* mice (Figure 5B, bottom panels), the proportion of IFN- γ -positive cells in CD4 $^{+}$ CD25 $^{+}$ regulatory T cells was signifi-

Figure 4. The concentration of IFN- γ is reduced in the lungs of GATA-3-overexpressing mice. The concentrations of IFN- γ (A), IL-4 (B), IL-5 (C), and IL-13 (D) in lung homogenates of wild-type mice (WT) and GATA-3-overexpressing mice (GATA-3) 7 days after intratracheal administration of 5 mg/kg bleomycin (bleo) or saline. $n = 6$ in each group. *Significant difference ($P < 0.05$) between WT and GATA-3 mice.

Figure 5. IFN- γ -producing CD4-positive T cells are decreased in the lungs of GATA-3-overexpressing mice. **A:** The proportion of IFN- γ -producing cells in lung CD4-positive T cells of wild-type mice (WT) and GATA-3-overexpressing mice (GATA-3) 7 days after intratracheal administration of 5 mg/kg bleomycin (bleo) or saline. $n = 3$ in each group. *Significant difference ($P < 0.05$) between the WT-bleo and GATA-3-bleo groups. **B:** The proportion of CXCR3-positive cells (top), CCR3-positive cells (middle), and CD25-positive cells (bottom) in CD4-positive T cells obtained from the lungs of wild-type mice (WT) and GATA-3-overexpressing mice (GATA-3) 7 days after intratracheal administration of 5 mg/kg bleomycin (bleo). $n = 3$ in each group. *Significant difference ($P < 0.05$) between the WT-bleo and GATA-3-bleo groups. **C:** The proportion of IFN- γ -producing cells in CXCR3-positive cells (top), in CCR3-positive cells (middle), and in CD25-positive cells (bottom) obtained from the lungs of wild-type mice (WT) and GATA-3-overexpressing mice (GATA-3) 7 days after intratracheal administration of 5 mg/kg bleomycin (bleo). $n = 3$ in each group. *Significant difference ($P < 0.05$) between the WT-bleo and GATA-3-bleo groups.

cantly higher in wild-type mice than in GATA-3-tg mice (Figure 5C, bottom panels). These results indicate that the production of IFN- γ was suppressed in lung CD4⁺ T cells, especially Th1 cells and regulatory T cells, of mice overexpressing the GATA-3 gene.

Regulation of TGF- β Expression by GATA-3

It has been demonstrated that the expression of TGF- β , a fibrogenic cytokine, is in part regulated by IFN- γ . We therefore evaluated the concentration of the active form of TGF- β in lung homogenates of wild-type mice and GATA-3-tg mice 7 days after the administration of bleomycin or saline. The protein level of the TGF- β active form was increased significantly in mice of both genotypes after bleomycin administration, compared with the corresponding control groups (Figure 6). However, the concentration of this form was significantly higher in GATA-

Figure 6. The level of the active form of TGF- β is increased in the lungs of GATA-3-overexpressing mice. The concentrations of TGF- β 1 in lung homogenates of wild-type mice (WT) and GATA-3-overexpressing mice (GATA-3) 7 days after intratracheal administration of 5 mg/kg bleomycin (bleo) or saline. $n = 4$ in each group. *Significant difference ($P < 0.05$) between the WT-bleo and GATA-3-bleo groups.

3-tg mice than in wild-type mice after bleomycin administration (Figure 6).

Effects of IFN- γ Supplementation

Because the concentration of IFN- γ was significantly lower in the lungs of GATA-3-tg mice than in those of wild-type mice, we next assessed whether supplementation with IFN- γ would reduce the development of bleomycin-induced pulmonary fibrosis. Mouse recombinant IFN- γ was administered continuously for 2 weeks via a miniosmotic pump beginning 1 day before the treatment with bleomycin or saline. We first evaluated the concentration of IFN- γ in lung homogenates 8 days after the initiation of IFN- γ administration to clarify whether the amount of exogenously administered IFN- γ was sufficient to increase the lung IFN- γ level. We confirmed that the lung IFN- γ level in GATA-3-tg mice was increased significantly to the level of wild-type mice by exogenous administration of IFN- γ (Figure 7A).

We next assessed the effects of exogenously administered IFN- γ on the development of pulmonary fibrosis using this model. Exogenous administration of IFN- γ significantly improved the survival rate of GATA-3-tg mice after bleomycin administration: 80% of IFN- γ -treated GATA-3-tg mice had survived at 28 days after bleomycin treatment (Figure 7B). No mice died in the saline-administered control groups, irrespective of whether or not they were treated with IFN- γ (Figure 7B). Lung histology showed that the degree of pulmonary fibrosis was much lower in IFN- γ -treated GATA-3-tg mice than in IFN- γ -untreated controls 28 days after bleomycin administration (Figure 7C). No abnormal alveolar architecture was observed in the lungs of the saline-administered control

groups, irrespective of whether or not they were simultaneously treated with IFN- γ (Figure 7C). Both the lung fibrotic score and the lung hydroxyproline content were also significantly reduced in IFN- γ -treated GATA-3-tg

mice to the level of wild-type mice 28 days after bleomycin administration (Figure 7, D and E, respectively). These results indicate that the supplementation of IFN- γ reduced the development of pulmonary fibrosis in mice overexpressing the GATA-3 gene.

Discussion

In the present study, we demonstrated for the first time that the development of bleomycin-induced pulmonary fibrosis is much more severe in GATA-3-tg mice than in wild-type mice of the same background. GATA-3 is a member of the GATA family of zinc-finger transcription factors, which bind the GATA consensus motif.^{26,27} It has been demonstrated that anti-sense GATA-3 inhibited the expression of all Th2 cytokine genes in the Th2 clone D10.¹⁸ In transgenic mice, elevated GATA-3 in CD4⁺ T cells caused Th2 cytokine gene expression in developing Th1 cells.¹⁸ GATA-3 has been reported to transactivate the IL-5 promoter with only limited effects on IL-4 gene transcription.^{18,21,28} It has also been reported that GATA-3 regulates the locus accessibility of the IL-4 and IL-13 genes with chromatin remodeling.^{29,30} These findings suggest that GATA-3 allows the expression of Th2 cytokines by functioning as a transcription factor as well as by modifying the chromatin structure of these cytokines.

In the present study, however, the lung IFN- γ level was characteristically suppressed after bleomycin administration in GATA-3-tg mice, compared with wild-type mice of the same background, while the pulmonary levels of Th2 cytokines such as IL-4, IL-5, and IL-13 were not different between them. We believe these findings are reasonable because several studies have demonstrated that GATA-3 not only transactivates Th2 cytokines but also suppresses Th1 cytokine expression. It was reported that GATA-3 significantly down-regulated IFN- γ production during *in vitro* Th1 differentiation of naïve CD4⁺ T cells through down-regulation of IL-12 receptor β 2 and IFN- γ production.^{19,31} In contrast, IFN- γ production in CD4⁺ T cells of GATA-3-deficient mice was increased even under Th2 condition.³² Furthermore, Kaminuma and colleagues³³ demonstrated that GATA-3 suppresses

Figure 7. Exogenous administration of IFN- γ attenuates the development of pulmonary fibrosis in GATA-3-overexpressing mice. **A:** The concentration of IFN- γ in the lungs of wild-type mice (WT), GATA-3-overexpressing mice (GATA-3), and GATA-3-overexpressing mice treated with mouse recombinant IFN- γ (GATA-3-IFN- γ) 7 days after intratracheal administration of 5 mg/kg bleomycin. $n = 4$ in each group. *Significant difference ($P < 0.05$) between the GATA-3 and GATA-3-IFN- γ groups. **B:** Survival of GATA-3-overexpressing mice treated with (squares) or without (circles) IFN- γ after intratracheal administration with 5 mg/kg bleomycin (bleo; closed) or saline (saline; open). $n = 20$ in each group. *Significant difference ($P < 0.05$) between the GATA-3-bleo and GATA-3-bleo-IFN- γ groups. **C:** Photomicrographs of lung tissues from GATA-3-overexpressing mice treated with or without IFN- γ 28 days after the intratracheal administration of 5 mg/kg bleomycin (bleo) or saline. Masson's trichrome stain. **D:** The fibrotic score (left) and lung hydroxyproline contents (right) of wild-type mice (WT), GATA-3-overexpressing mice (GATA-3), and GATA3-overexpressing mice treated with IFN- γ (GATA-3+IFN- γ) 28 days after intratracheal administration of 5 mg/kg bleomycin. $n = 4$ in each group. *Significant difference ($P < 0.05$) between WT and GATA-3 mice. **Significant difference ($P < 0.05$) between the GATA-3 and GATA-3-IFN- γ groups. Scale bar = 100 μ m.

IFN- γ gene transcription via the down-regulation of Stat4. Although it remains unclear why GATA-3 overexpression did not affect Th2 cytokine production in the present model, the genetic background of the parental strain may be an important factor for Th1/Th2 responses. C57BL/6 mice are known as a Th1 responder strain. C57BL/6 mice produce a large amount of IFN- γ by several stimuli including bleomycin.³⁴ It has also been reported that ectopic expression of GATA-3 leads to the inhibition of IFN- γ production under Th1-polarizing conditions.^{17,19,20} Thus, GATA-3 overexpression suppresses Th1 cytokine production rather than up-regulating Th2 cytokine production under a Th1-dominant condition, such as in bleomycin-treated C57BL/6 mice.

In the present study, exogenous administration of IFN- γ to GATA-3-*tg* mice improved the degree of pulmonary fibrosis to the level of wild-type mice. Our recent study demonstrated that treatment of C57BL/6 mice with α -galactosylceramide, a selective ligand for natural killer T cells, attenuated the development of bleomycin-induced pulmonary fibrosis.³⁵ The protective effect of α -galactosylceramide was associated with an increase in the pulmonary IFN- γ level. In addition, neutralization of endogenously synthesized IFN- γ with a specific antibody abrogated the protective effects of α -galactosylceramide. Similarly, Kim and colleagues³⁶ have demonstrated that natural killer T cells attenuated pulmonary fibrosis by directly producing IFN- γ . Previous studies have also demonstrated that IFN- γ exerts anti-fibrotic effects in experimental animals, including a bleomycin-induced pulmonary fibrosis model.^{12,13} It is therefore possible to say that IFN- γ plays a key role in the susceptibility of GATA-3-*tg* mice to bleomycin-induced pulmonary fibrosis.

To generate GATA3-*tg* mice, we inserted a full-length murine GATA-3 cDNA into a VA CD2 transgene cassette.²² The VA vector has been reported to directly express the inserted cDNA in all single-positive mature T cells of transgenic mice.³⁷ Because GATA-3 is a T-cell-specific transcription factor, transgenic mice are thought to be adequate for use in evaluating T-cell-mediated regulation of pulmonary fibrosis. In the present study, intracellular cytokine analysis revealed that the suppression of IFN- γ production in GATA-3-*tg* mice occurred in CD4⁺ T cells, especially Th1 cells and regulatory T cells. These findings suggest that the overexpressed GATA-3 gene actually acts on CD4⁺ T cells in the present model.

Whether or not T cells contribute to the pathogenesis of pulmonary fibrosis is still controversial both in humans and in experimental animals. The depletion of T cells by antibodies has been shown to inhibit the development of bleomycin-induced pulmonary fibrosis.⁷ Bleomycin-induced pulmonary inflammation and fibrosis were not observed in athymic nude mice.⁸ These findings suggest that T cells participate in the pathophysiology of fibrosis in the bleomycin model. Another study, however, has demonstrated that bleomycin-induced pulmonary fibrosis occurred in nude mice and SCID mice that lack T cells.^{38,39} In patients with idiopathic pulmonary fibrosis, lymphocytosis is not a characteristic feature. However, it has been shown that a significant number of both CD4⁺ and CD8⁺ T cells are infiltrated into the lung parenchyma

of patients with idiopathic pulmonary fibrosis.⁴⁰ Analysis of the CD4⁺ T-cell subpopulation has shown that patients with idiopathic pulmonary fibrosis have a significantly lower CXCR3 and a higher CCR4 expression on bronchoalveolar lavage CD4⁺ T cells.⁴¹ Moreover, treatment of these patients with corticosteroids results in higher CXCR3 and lower CCR4 expression compared with those in untreated patients. These findings demonstrate that an imbalance in CXCR3/CCR4, that is, an imbalance of Th1/Th2, actually occurs in the lungs of patients with idiopathic pulmonary fibrosis.

Some researchers hypothesize that idiopathic pulmonary fibrosis is a disease in which parenchymal fibrosis is directly caused by chronic inflammation.^{42,43} An early hypothesis suggested that an unidentified insult initiated a cycle of chronic inflammatory injury leading to fibrosis. Although corticosteroid and cytotoxic agents have been used in patients with idiopathic pulmonary fibrosis to interrupt the inflammatory cascade before irreversible tissue injury occurred, it is now clear that these anti-inflammatory therapies provide no benefit. A newer hypothesis suggests that the pulmonary fibrosis results from sequential acute lung injury.⁴⁴ The resultant wound-healing response to this lung injury culminates in pulmonary fibrosis. The fibrotic response is modified by several interacting factors, including the genetic background, environmental inflammatory triggers, and the predominant inflammatory phenotype (Th1 or Th2). Although the Th2 and Th1 phenotypes are not as well defined in idiopathic pulmonary fibrosis as they are in asthma and animal models, their potential importance is one rationale for trials of immunomodulators such as IFN- γ in attempts to switch the inflammatory responses to a more Th1-like phenotype.

In the present study, surprisingly, the production of IFN- γ in CD4⁺CD25⁺ regulatory T cells was decreased in the lungs of GATA-3-*tg* mice. Regulatory T cells are a small subset of CD4⁺ T cells that exhibit potent immunosuppressive properties. Because recent studies suggest that regulatory T cells protect against the development of Th2 diseases such as allergic and asthmatic diseases,⁴⁵ it is likely that regulatory T cells negatively regulate the development of lung fibrosis. The development and function of CD4⁺CD25⁺ regulatory T cells are under the control of the transcription factor Foxp3, which is highly expressed in these cells.^{46,47} The contribution of GATA-3 to the differentiation of regulatory T cells and to the production of IFN- γ in these cells should be examined in future studies.

The potential of IFN- γ as a treatment for idiopathic pulmonary fibrosis has not been established, and some alternative strategies are needed, because of the recent failure in the IFN- γ treatment clinical trials.⁴⁸ Although there is currently no direct evidence that GATA-3 plays a role in the pathogenesis of human pulmonary fibrosis, Th1/Th2 cell regulation by transcription factors might be a useful alternative approach for the treatment of pulmonary fibrosis. Our observation that the overexpression of GATA-3 exacerbated the development of pulmonary fibrosis by inducing a relative Th2 bias suggests that inhibiting the activation of GATA-3 might be useful as a

therapeutic strategy for pulmonary fibrosis. On the other hand, the transcription factor T-bet is thought to be a key regulator of Th1 differentiation. T-bet expression is strongly correlated with IFN- γ expression and is specifically up-regulated in primary Th cells that differentiate along the Th1 but not the Th2 pathways.⁴⁹ T-bet-deficient mice have been shown to develop spontaneous airway changes consistent with human asthma, which is a Th2 model disease.⁵⁰ Finotto and colleagues⁵¹ have recently demonstrated that T-bet negatively regulates lung remodeling, such as subepithelial collagen deposition and myofibroblast transformation, by controlling the interaction with IL-13 and TGF- β in the asthmatic airway. It is therefore likely that the regulation of T-bet gene expression influences the development of pulmonary fibrosis. Therefore, we are currently investigating the protective effects of T-bet overexpression on the development of bleomycin-induced pulmonary fibrosis. We suspect that Th1/Th2 regulation by these transcription factors has an advantage over the systemic administration of IFN- γ for the treatment of pulmonary fibrosis for the following reasons: the transactivation of the target proteins occurs specifically in T cells, and thus the systemic side effects can be reduced; and it is expected that Th1/Th2 regulation will not only up-regulate IFN- γ production but also suppress the production of Th2 cytokines that enhance fibrotic processes, such as IL-4 and IL-13.

Idiopathic pulmonary fibrosis is a lethal disorder for which efficacious therapeutic approaches are not readily available. Although transcription factor regulation therapy cannot be used currently for the treatment of pulmonary fibrosis, we believe that this study may provide the possibility of new therapies for the treatment of pulmonary fibrosis.

References

- Ward PA, Hunninghake GW: Lung inflammation and fibrosis. *Am J Respir Crit Care Med* 1998, 157:S123–S129
- Adamson IY, Bowden DH: The pathogenesis of bleomycin-induced pulmonary fibrosis in mice. *Am J Pathol* 1974, 77:185–197
- Snider GL, Hayes JA, Korthy AL: Chronic interstitial pulmonary fibrosis produced in hamsters by endotracheal bleomycin: pathology and stereology. *Am Rev Respir Dis* 1978, 117:1099–1108
- Sleijfer S: Bleomycin-induced pneumonitis. *Chest* 2001, 120:617–624
- Chandler DB, Hyde DM, Giri SN: Morphometric estimates of infiltrative cellular changes during the development of bleomycin-induced pulmonary fibrosis in hamsters. *Am J Pathol* 1983, 112:170–177
- Thrall RS, Barton RW: A comparison of lymphocyte populations in lung tissue and in bronchoalveolar lavage fluid of rats at various times during the development of bleomycin-induced pulmonary fibrosis. *Am Rev Respir Dis* 1984, 129:279–283
- Piguet PF, Collart MA, Grau GE, Kapanci Y, Vassalli P: Tumor necrosis factor/cachectin plays a key role in bleomycin-induced pneumonopathy and fibrosis. *J Exp Med* 1989, 170:655–663
- Schrier DJ, Phan SH, McGarry BM: The effects of the nude (nu/nu) mutation on bleomycin-induced pulmonary fibrosis: a biochemical evaluation. *Am Rev Respir Dis* 1983, 127:614–617
- Liu X, Kohyama T, Wang H, Zhu YK, Wen FQ, Kim HJ, Romberger DJ, Rennard SI: Th2 cytokine regulation of type I collagen gel contraction mediated by human lung mesenchymal cells. *Am J Physiol* 2002, 282:L1049–L1056
- Sempowski GD, Derdak S, Phipps RP: Interleukin-4 and interferon-gamma discordantly regulate collagen biosynthesis by functionally distinct lung fibroblast subsets. *J Cell Physiol* 1996, 167:290–296
- Postlethwaite AE, Holness MA, Katai H, Raghow R: Human fibroblasts synthesize elevated levels of extracellular matrix proteins in response to interleukin 4. *J Clin Invest* 1992, 90:1479–1485
- Gurujeyalakshmi G, Giri SN: Molecular mechanisms of antifibrotic effect of interferon- γ in bleomycin-mouse model of lung fibrosis: downregulation of TGF- β and procollagen I and III gene expression. *Exp Lung Res* 1995, 21:791–808
- Hyde DM, Henderson TS, Giri SN, Tyler NK, Stovall MY: Effect of murine gamma interferon on the cellular responses to bleomycin in mice. *Exp Lung Res* 1988, 14:687–704
- Saito A, Okazaki H, Sugawara I, Yamamoto K, Takizawa H: Potential action of IL-4 and IL-13 as fibrogenic factors on lung fibroblasts in vitro. *Int Arch Allergy Immunol* 2003, 132:168–176
- Abbas AK, Murphy KM, Sher A: Functional diversity of helper T lymphocytes. *Nature* 1996, 383:787–793
- Liblau RS, Singer SM, McDevitt HO: Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. *Immunol Today* 1995, 16:34–38
- Zhang DH, Cohn L, Ray P, Bottomly K, Ray A: Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. *J Biol Chem* 1997, 272:21597–21603
- Zheng DH, Flavell RA: The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 1997, 89:587–596
- Ouyang W, Ranganath SH, Weindel K, Bhattacharya D, Murphy TL, Sha WC, Murph KM: Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. *Immunity* 1998, 9:745–755
- Usui T, Nishikomori R, Kitani A, Strober W: GATA-3 suppresses Th1 development by downregulation of Stat4 and not through effects on IL-12R β 2 chain or T-bet. *Immunity* 2003, 18:415–428
- Zhang DH, Yang L, Ray A: Differential responsiveness of the IL-5 and IL-4 genes to transcription factor GATA-3. *J Immunol* 1998, 161:3817–3821
- Yoh K, Shibuya K, Morito N, Nakano T, Ishizaki K, Shimohata H, Nose M, Izui S, Shibuya A, Koyama A, Engel JD, Yamamoto M, Takahashi S: Transgenic overexpression of GATA-3 in T lymphocytes improved autoimmune glomerulonephritis in mice with a BXSB/MpJ-Yaa genetic background. *J Am Soc Nephrol* 2003, 14:2494–2502
- Ashcroft T, Simpson JM, Timbrell V: Simple method of estimating severity of pulmonary fibrosis on a numerical scale. *J Clin Pathol* 1988, 41:467–470
- Woessner LF: The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys* 1961, 93:440–447
- Murphy E, Shibuya K, Hosken N, Openshaw P, Maino V, Davis K, Murphy K, O'Garra A: Reversibility of T helper 1 and 2 populations is lost after long-term stimulation. *J Exp Med* 1996, 183:901–913
- Yamamoto M, Ko LJ, Leonard MW, Beug H, Orkin SH, Engle JD: Activity and tissue-specific expression of the transcription factor NF-E1 multigene family. *Genes Dev* 1990, 4:1650–1662
- Ko LJ, Yamamoto M, Leonard MW, George KM, Ting P, Engel JD: Murine and human T-lymphocyte GATA-3 factors mediate transcription through a cis-regulatory element within the human T-cell receptor α gene enhancer. *Mol Cell Biol* 1991, 11:2778–2784
- Ranganath S, Ouyang W, Bhattacharya D, Sha WC, Grupe A, Peltz G, Murphy KM: GATA-3-dependent enhancer activity in IL-4 gene regulation. *J Immunol* 1998, 161:3822–3826
- Takemoto N, Kamogawa Y, Lee HJ, Kuruta H, Arai K, O'Garra A, Arai N, Miyataka S: Chromatin remodeling at the IL4/IL13 intergenic regulatory region for Th2-specific cytokine gene cluster. *J Immunol* 2000, 165:6687–6691
- Lee GR, Field PE, Flavell RA: Regulation of IL-4 gene expression by distal regulatory elements and GATA-3 at the chromatin level. *Immunity* 2001, 14:447–459
- Ferber IA, Lee HJ, Zonin F, Heath V, Mui A, Arai N, O'Garra A: GATA-3 significantly downregulates IFN- γ production from developing Th1 cells in addition to inducing IL-4 and IL-5 levels. *Clin Immunol* 1999, 91:134–144
- Pai SY, Truitt ML, Ho IC: GATA-3 deficiency abrogates the development and maintenance of T helper type 2 cells. *Proc Natl Acad Sci USA* 2004, 101:1993–1998
- Kaminuma O, Kitamura F, Kitamura N, Miyagishi M, Taira K,

- Yamamoto K, Miura O, Miyatake S: GATA-3 suppresses IFN- γ promoter activity independently of binding to cis-regulatory element. *FEBS Lett* 2004, 570:63–68
34. Gur I, Or R, Segel MJ, Shriki M, Izbicki G, Breuer R: Lymphokines in bleomycin-induced lung injury in bleomycin-sensitive C57BL/6 and -resistant BALB/c mice. *Exp Lung Res* 2000, 26:521–534
35. Kimura T, Ishii Y, Morishima Y, Shibuya A, Shibuya K, Taniguchi M, Mochizuki M, Hegab AE, Sakamoto T, Nomura A, Sekizawa K: Treatment with α -galactosylceramide attenuates the development of bleomycin-induced pulmonary fibrosis. *J Immunol* 2004, 172:5782–5789
36. Kim JH, Kim HY, Kim S, Chung JH, Park WS, Chung DH: Natural killer T (NKT) cells attenuate bleomycin-induced pulmonary fibrosis by producing interferon- γ . *Am J Pathol* 2005, 167:1231–1241
37. Zhumabekov T, Corbella P, Tolaini M, Kioussis D: Improved version of a human CD2 minigene based vector for T cell-specific expression in transgenic mice. *J Immunol Methods* 1995, 185:133–140
38. Szapiel SV, Elson NA, Fulmer JD, Hunninghake GW, Crystal RG: Bleomycin-induced interstitial pulmonary disease in the nude, athymic mouse. *Am Rev Respir Dis* 1979, 120:893–899
39. Helene M, Lake-Bullock V, Zhu J, Hao H, Cohen DA, Kaplan AM: T cell independence of bleomycin-induced pulmonary fibrosis. *J Leukoc Biol* 1999, 65:187–195
40. Papiris SA, Kollintza A, Kitsanta P, Kapotsis G, Karatza M, Milic-Emili J, Roussos C, Daniil Z: Relationship of BAL and lung tissue CD4+ and CD8+ T lymphocytes, and their ratio in idiopathic pulmonary fibrosis. *Chest* 2005, 128:2971–2977
41. Pignatti P, Brunetti G, Moretto D, Yacoub MR, Fiori M, Balbi B, Balestrino A, Cervio G, Nava S, Moscato G: Role of the chemokine receptors CXCR3 and CCR4 in human pulmonary fibrosis. *Am J Respir Crit Care Med* 2006, 173:310–317
42. Mason RJ, Schwarz MI, Hunninghake GW, Musson RA: Pharmacological therapy for idiopathic pulmonary fibrosis: past, present, and future. *Am J Respir Crit Care Med* 1999, 160:1771–1777
43. Katzenstein AL, Myers JL: Idiopathic pulmonary fibrosis: clinical relevance of pathologic classification. *Am J Respir Crit Care Med* 1998, 157:1301–1315
44. Gross TJ, Hunninghake GW: Idiopathic pulmonary fibrosis. *N Engl J Med* 2001, 345:517–525
45. Hawrylowicz CM: Regulatory T cells and IL-10 in allergic inflammation. *J Exp Med* 2005, 202:1459–1463
46. Fontenot JD, Gavin MA, Rudensky AY: Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 2003, 4:330–336
47. Hori S, Nomura T, Sakaguchi S: Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003, 299:1057–1061
48. Raghu G, Brown KK, Bradford WZ, Starko K, Noble PW, Schwartz DA, King Jr TE: Idiopathic Pulmonary Fibrosis Study Group. A placebo-controlled trial of interferon γ -1b in patients with idiopathic pulmonary fibrosis. *N Engl J Med* 2004, 350:125–133
49. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH: A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 2000, 100:655–669
50. Finotto S, Neurath MF, Glickman JN, Qin S, Lehr HA, Green FHY, Ackerman K, Haley K, Galle PR, Szabo SJ, Drazen JM, De Sanctis GT, Glimcher LH: Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science* 2002, 295:336–338
51. Finotto S, Hausding M, Doganci A, Maxeiner JH, Lehr HA, Luft C, Galle PR, Glimcher LH: Asthmatic changes in mice lacking T-bet are mediated by IL-13. *Int Immunol* 2005, 17:993–1007