Cardiovascular, Pulmonary and Renal Pathology

Natural Killer T (NKT) Cells Attenuate Bleomycin-Induced Pulmonary Fibrosis by Producing Interferon-γ

Pulmonary fibrosis is a progressive illness characterized by interstitial fibrosis. Although the precise mechanism for pulmonary fibrosis is not completely understood, an immune response involving interferon (IFN)-γ appears to play a role. Therefore, we examined the functional roles of natural killer T (NKT) cells, which produce IFN-γ and interleukin-4 on activation, in bleomycin-induced pulmonary fibrosis. In NKT cell-deficient mice, pulmonary fibrosis was worse in terms of histology, hydroxyproline levels, and mortality than in control mice. The transforming growth factor (TGF)-β1 levels were higher in the lung after injecting bleomycin, and blockade of TGF-β1 by neutralizing monoclonal antibody attenuated the pulmonary fibrosis in CD1d−/− mice. In contrast, the production of IFN-γ was reduced in lungs from CD1d−/− mice. Moreover, the adoptive transfer of NKT cells into CD1d−/− mice increased IFN-γ and reduced TGF-β1 production, attenuating pulmonary fibrosis. An in vitro assay demonstrated that IFN-γ was involved in suppressing TGF-β1 production in cells collected from bronchoalveolar lavage. The adoptive transfer of NKT cells from IFN-γ−/− mice did not reverse pulmonary fibrosis or TGF-β1 production in lungs of CD1d−/− mice whereas NKT cells from B6 control mice attenuated fibrosis and reduced TGF-β1 production. In conclusion, IFN-γ-producing NKT cells play a novel anti-fibrotic role in pulmonary fibrosis by regulating TGF-β1 production. 

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attenuated the bleomycin-induced pulmonary fibrosis and its protective effects were associated with an increase in the IFN-γ level and a decrease in the transforming growth factor (TGF)-β level in the lung, suggesting that α-GalCer might be a useful therapeutic reagent to reduce fibrosis. Therefore, it was proposed that NKT cells play critical roles in the bleomycin-induced pulmonary fibrosis. However, the mechanism by which NKT cells play this critical role in the development of bleomycin-induced pulmonary fibrosis is unclear.

This study examined the functional roles of NKT cells in bleomycin-induced pulmonary fibrosis. The results show that NKT cells play a novel anti-fibrotic role by producing IFN-γ at approximately day 7 rather than an earlier time point in bleomycin-induced pulmonary fibrosis, which contributes to the suppression of TGF-β production in the lung.

**Materials and Methods**

**Mice and Bleomycin-Induced Pulmonary Fibrosis Model**

C57BL/6 mice were purchased from DaeHan Biolink (Seoul, Korea). CD1d<sup>-/-</sup> mice (C57BL/6 background) were a generous gift from Dr. Hua Gu (Columbia University, New York, NY). Ja<sup>18<sup>-/-</sup> (C57BL/6 background) and RAG<sup>-/-</sup> Vα14<sup>19</sup> Vβ8.2<sup>19</sup> mice were a generous gift from Dr. M. Taniguchi (Chiba University, Chiba, Japan). IFN-γ<sup>-/-</sup> and IL-4<sup>-/-</sup> mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Male mice, 8 to 10 weeks of age, were used in all of the experiments. These mice were bred and maintained under specific pathogen-free conditions at the Clinical Research Institute, Seoul National University Hospital. All animal experiments were performed after receiving approval of the Institutional Animal Care and Use Committee of Clinical Research Institute, Seoul National University Hospital. To induce pulmonary fibrosis, the mice were anesthetized with 2,2,2-tribromomethanol (Sigma-Aldrich, St. Louis, MO) and injected with 2 mg/kg of bleomycin (Nippon Kayaku, Tokyo, Japan) in phosphate-buffered saline (PBS) (50 μl) via intratracheal instillation in all experiments except for those experiments evaluating the survival rates of the mice. The mice were injected with 3 mg/kg of bleomycin to evaluate clinical outcomes.

**Hydroxyproline Assay**

The total hydroxyproline level of the lung was measured on day 21 after the bleomycin injection. The whole lung was excised, homogenized in PBS (2 ml), and dried for 6 hours by vacuum dryer. The samples were added to 1 ml of 6 N HCl for 12 hours at 110°C and then filtered. Aliquots (50 μl) of the samples were then examined by adding 50 μl of citrate acetate buffer and 1 ml of chloramines T solution followed by a reaction with Erlich’s solution at 65°C for 15 minutes. The absorbance was measured at 550 nm using a spectrophotometer (DU650; Beckman). The increased hydroxyproline contents in the lungs of experimental groups were compared with the amount of hydroxyproline of the lung from untreated B6 mice. The value of the B6 control group was set to 1 and the relative increased values of hydroxyproline in each group were calculated. The results were statistically analyzed using analysis of variance and presented as percentage.

**Bronchoalveolar Lavage (BAL) and Fluorescence-Activated Cell Sorting Analysis**

The mice were euthanized via CO<sub>2</sub> asphyxiation. The trachea was cannulated, and the lung was lavaged five times with 0.7 ml of cold PBS. The BAL fluid was centrifuged at 1500 rpm for 10 minutes at 4°C, and the supernatant was removed. The total BAL cells were counted using a hemocytometer, and incubated for 15 minutes on ice in FcγRII/III blockade. After washing, the cells were stained in a 200-μl total volume with a 1-μg combination of the following monoclonal antibodies (mAbs): anti-CD8, CD4, CD25, CD69, NK1.1, F4/80, TCR-β, which were purchased from BD Pharmingen (San Diego, CA). The cells were collected using cytospin (10 minutes at 55 × g) and stained with hematoxylin and eosin (H&E) to evaluate the number of polymorphonuclear leukocytes in the BAL. For identification of CD1d-restricted NKT cells, the total BAL cells were preincubated with mAb (24G2, BD Pharmingen) against the Fcγ receptors, washed, and incubated with anti-TCR-β mAb (BD Pharmingen) and α-GalCer-loaded dimer (dimer X I: recombinant soluble mouse CD1d:Ig fusion protein; BD Pharmingen) conjugated with phycoerythrin-streptavidin for 1 hour at 37°C. The cells were washed and analyzed on a FACSCalibur (Becton Dickinson, San Diego, CA).

**Adoptive Transfer Experiment**

After sacrifice, the livers were homogenized and resuspended in a loading buffer (PBS plus 10% fetal bovine serum and 1 mmol/L ethylenediamine tetraacetic acid) and overlaid onto lympholyte-M (Cedarlane, Ontario, Canada). After centrifugation for 20 minutes at 900 × g at 25°C, the liver mononuclear cells were isolated from the interface and washed with PBS. The cells obtained were stained with phycoerythrin-conjugated anti-NK1.1 (BD Pharmingen) and Cy-conjugated anti-TCR-β (BD Pharmingen). The stained cells were then sorted by FACStar Plus using CellQuest software, and the purity of the sorted cells was >95%. The splenocyte suspensions of RAG<sup>-/-</sup> Vα14<sup>19</sup> Vβ8.2<sup>19</sup> mice and Ja<sup>18<sup>-/-</sup> mice were prepared and depleted of red blood cells using a RBC lysis buffer (Sigma-Aldrich, St. Louis, MO) and washed with PBS. NKT cells (3 × 10<sup>5</sup> per mouse) or splenocytes (1 × 10<sup>7</sup> per mouse) were adoptively transferred via an intravenous injection.
Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Real-Time PCR Analysis

For quantitative real-time PCR, the total RNA was isolated from the whole lung homogenates using an RNeasy kit (Qiagen, Courtaboeuf, France) according to the manufacturer’s instructions. The contaminated genomic DNA was digested with DNase I (Qiagen, Seoul, Korea) and PCR was then performed. Briefly, 1 μg of cDNA was amplified in the presence of a TaqMan universal master mix (Perkin-Elmer Biosystems), gene-specific TaqMan probe, the forward and reverse primers, and water. The GAPDH-specific TaqMan probe and forward and reverse primers were used as the endogenous control. The gene-specific PCR products were measured using an Applied Biosystems 7500 sequence detection system (Perkin-Elmer Biosystems). The results for each cytokine were normalized with respect to the GAPDH expression level. The reactions in each group were performed in triplicate. For real-time PCR, the following primers were synthesized by Applied Biosystems (Foster City, CA): GAPDH: TGCACACCAACTGCTTA (forward), GGA-TGCAGGGATGATGT (reverse), and CAGGAAGCTTG-GGTGCCCCC-VIC; IFN-γ: AGCAACAGCAAGCGA-AAA (forward), CTGGACCTGTGGTTGTTGTTGA (reverse), and CTCAAACTTGGCAATCTCAGATCGACTC-TAM-RA; and TGF-β1: GCAACATGGAACACTCAGACACAA (reverse), and ACGTGAACCATCGTCTGCCCC-TAM-RA; and IL-4: FAM-CTC CAG (reverse); FasL: ATCCCTCTGGAATGGGAAGACTCATGCCAAC (forward) and ACGTGAACCATAAGACTCATGACCCAGTG (reverse); Fas: TTGCTGTCAACTGGCAGGATCTG (forward) and AC-CTTTTCAGCTGTATAGGG (reverse); TGF-β1: GAGCCTTAGTTTGGGCTGG (forward) and GAGCCTTAGTTTGGGCTGG (reverse); tumor necrosis factor-α: GGCAAGGATCTG (reverse); and ACATGCCACCAACTGCTTA (forward), GGA-TGCAGGGATGATGT (reverse), and CAGGAAGCTTG-GGTGCCCCC-VIC. For RT-PCR, the following primers were synthesized by Genotech Corp. (Daejeon, Korea): β-actin: CC-CAACTTGTATGATGAAAG (forward) and TTTCGGTGAAGTAACTG (reverse); IFN-γ: AGCCGGCT-GACTGAACACTCAGATT (forward) and GTACAG-ACTGGTATAGG (reverse); TGF-β1: TGACAT-GGAACCTGCTGAA (forward) and GAGCCTTAGTTTGGGCTGG (reverse); and ACATCCACCAACTGCTTA (forward), GGA-TGCAGGGATGATGT (reverse), and CAGGAAGCTTG-GGTGCCCCC-VIC. The hybridomas for anti-TGF-β mAb (clone 1D11.16.8) and anti-IFN-γ mAb (clone R4-6A2) were purchased from the American Type Culture Collection. The mAbs were purified using a protein G column (Amersham Bioscience, Sweden). Five hundred μg of each mAb was injected via intravenously 1 day before, and 4, 8, 12, and 16 days after the administration of bleomycin.

Cytokine Neutralization in Vivo Using Blocking mAb

The hybridomas for anti-TGF-β mAb (clone 1D11.16.8) and anti-IFN-γ mAb (clone R4-6A2) were purchased from the American Tissue Cell Culture. The mAbs were purified and used as a protein G column (Amersham Bioscience, Sweden). Five hundred μg of each mAb was injected via intravenously 1 day before, and 4, 8, 12, and 16 days after the administration of bleomycin.

Histology and Pathological Scoring

The mice were euthanized and perfused with saline via the right ventricle. The lungs were inflated with 1 ml of 10% neutral buffered formalin and fixed for 24 hours. Routine histological techniques were used to paraaffin-embed the entire lung. The sections were cut, mounted on slides, and stained with H&E. The mice were characterized using a scoring method as previously described. The pathological scores were defined as follows: 0, no lung abnormality; 1, the presence of inflammation and fibrosis involving <25% of the lung; 2, lesions involving 25 to 50% of the lung; and 3, lesions involving >50% of the lung. The mean of the pathological scores for five mice was determined for each group.

Statistics

Statistical significance was analyzed using the Prism 3.0 program. Student’s t-tests were used to compare two groups. The survival data were analyzed using the log-rank test. P values <0.05 were considered significant. In terms of increased contents of hydroxyproline in the lungs, the one-way analysis of variance statistical analysis was performed. The results are expressed as a mean ± SEM.

Results

NKT Cell-Deficient Mice Aggravate Bleomycin-Induced Pulmonary Fibrosis

To investigate the functional roles of NKT cells during the development of bleomycin-induced pulmonary fibrosis, we examined the kinetics of infiltrated total immune cells and NKT cells in the BAL fluid. In wild-type B6 mice, the...
number of immune cells in the BAL fluids increased 4 days after the intratracheal administration of bleomycin and reached a peak level on day 7 (Figure 1A). NKT cells, recognized by the α-GalCer/CD1d dimer, were detected in BAL fluid whereas NKT cells were rarely found in spleen of wild-type B6 mice. As shown in Figure 2D, most of the NKT cells were adoptively transferred into CD1d<sup>−/−</sup> mice compared with wild-type B6 mice. All CD1d<sup>−/−</sup> mice and 70% of Jα18<sup>−/−</sup> mice died within 2 weeks after administering the high dose (3 mg/kg) of bleomycin whereas wild-type B6 mice were still alive for up to 3 weeks (Figure 2E). Overall, these findings suggest that NKT cells in mice play a suppressive role in the bleomycin-induced acute lung injury and fibrosis.

Adoptive Transfer of NKT Cells Attenuates Pulmonary Fibrosis in NKT Cell-Deficient Mice

To demonstrate that the lack of NKT cells specifically caused an increase in pulmonary fibrosis in CD1d<sup>−/−</sup> mice, either splenocytes of RAG<sup>−/−</sup> Vα<sub>14</sub>49 Vβ<sub>8.2</sub>9 mice (Figure 3A) or NKT cells from normal B6 mice (Figure 3B) were adoptively transferred into CD1d<sup>−/−</sup> mice and the contents of hydroxyproline were measured in these mice. Splenocytes of Jα18<sup>−/−</sup> mice were adoptively transferred as the control. RAG<sup>−/−</sup> Vα<sub>14</sub>49 Vβ<sub>8.2</sub>9 mice contain NKT cells expressing Vα<sub>14</sub>49 Jα18 TCR in vivo. 28–30 the kinetics of NKT cell infiltration in the lung was similar to that of the total inflammatory cells (Figure 1B). The total cell numbers were counted and subset analysis for NK cells, T cells, macrophages, and polymorphonuclear leukocytes (PMN) were performed in the BAL fluids from wild-type B6 and CD1d<sup>−/−</sup> mice 7 days after the bleomycin injection. The total number of cells and the number of each subset of immune cells in the BAL fluid were similar in B6 and CD1d<sup>−/−</sup> mice of bleomycin-induced pulmonary fibrosis model (Figure 1D). A recent study suggested that NKT cells might contribute to activating NK1.1<sup>+</sup> TCR-β<sup>+</sup> conventional T cells in lung during bleomycin-induced pulmonary fibrosis. 25 Therefore, the activation status of conventional T cells in the lung was analyzed by evaluating the expression level of activation markers such as CD25 and CD69. However, the expression levels of CD25 and CD69 on the conventional T cells of the BAL from B6 mice were similar to those from CD1d<sup>−/−</sup> mice in bleomycin-induced pulmonary fibrosis (Figure 1E). To determine whether the depletion of NKT cells in vivo contributes to the development of pulmonary fibrosis, pulmonary fibrosis was induced in CD1d<sup>−/−</sup> and Jα18<sup>−/−</sup> mice by an intratracheal injection of bleomycin. Bleomycin induced multifocal fibrotic pulmonary lesions with thickened alveolar septa, collapses of alveolar spaces, and massive infiltrations of lymphocytes in the interstitium of wild-type mice as estimated 21 days after the injection of bleomycin (Figure 2A). The histological scorings of the fibrotic lesions revealed significant aggravation of bleomycin-induced pulmonary fibrosis in CD1d<sup>−/−</sup> mice compared with wild-type B6 mice (Figure 2B). Moreover, the content of hydroxyproline, a collagen component, in the lung was significantly higher in CD1d<sup>−/−</sup> and Jα18<sup>−/−</sup> mice than in wild-type mice, as estimated 21 days after administering bleomycin (Figure 2C). These findings indicate that the bleomycin-induced pulmonary fibrosis is significantly increased in NKT cell-deficient mice as compared with wild-type B6 mice.

The biological significance of bleomycin-induced pulmonary fibrosis was assessed by measuring the body weight and estimating the survival rate of CD1d<sup>−/−</sup>, Jα18<sup>−/−</sup>, and wild-type B6 mice. As shown in Figure 2D, the weight losses accelerated significantly in CD1d<sup>−/−</sup> and Jα18<sup>−/−</sup> mice compared with wild-type B6 mice. All CD1d<sup>−/−</sup> mice and 70% of Jα18<sup>−/−</sup> mice died within 2 weeks after administering the high dose (3 mg/kg) of bleomycin whereas wild-type B6 mice were still alive for up to 3 weeks (Figure 2E). Overall, these findings suggest that NKT cells in mice play a suppressive role in the bleomycin-induced acute lung injury and fibrosis.
tents in the lung of CD1d\(^{-/-}\) mice administrated intratra-
cheally with bleomycin (Figure 3, A and B). These find-
ings indicate that adoptively transferred NKT cells in
CD1d\(^{-/-}\) mice attenuated the bleomycin-induced pulmo-
nary fibrosis.

NKT Cells Attenuate Pulmonary Fibrosis by
Suppression of the Production of TGF-\(\beta\)

To investigate the mechanism by which an absence of
NKT cells aggravated bleomycin-induced pulmonary
fibrosis in CD1d\(^{-/-}\) mice, the expression pattern of
various cytokines in the lungs of B6, J\(\alpha\)18\(^{-/-}\), and
CD1d\(^{-/-}\) mice was examined using RT-PCR (Figure
4A). Among the cytokines tested, the significantly
higher TGF-\(\beta\)1 transcription was detected in CD1d\(^{-/-}\)
mice 7 and 14 days after bleomycin injection whereas
it was barely detectable in wild-type B6. In contrast,
wild-type B6 mice produced a significant level of IFN-\(\gamma\)
transcription in the bleomycin-treated lung whereas
there was little IFN-\(\gamma\) transcription in the lung of
CD1d\(^{-/-}\) mice treated with bleomycin. Furthermore,
real-time PCR showed that the induction of TGF-\(\beta\)1
mRNA increased significantly and the induction of
IFN-\(\gamma\) mRNAs were reduced in the lung of CD1d\(^{-/-}\)
mice treated with bleomycin (Figure 4C). Kinetic anal-
ysis of IFN-\(\gamma\) and TGF-\(\beta\)1 production in the BAL fluid
and lung tissues revealed that the amounts of IFN-\(\gamma\)
were high on days 1 and 7 after the bleomycin injection
into B6 mice whereas CD1d\(^{-/-}\) mice maintained low

Figure 2. NKT cell-deficient mice aggravate bleomycin-induced pulmonary fibrosis. A: The lungs were removed from B6 and CD1d\(^{-/-}\) mice on day 21 after injecting bleomycin (right) or PBS (left), and paraffin sections were stained with H&E. The pictures represent 1 of 10 mice in each group. B: Fibrotic responses in the lungs of B6 and CD1d\(^{-/-}\) mice were graded according to the criteria described in Materials and Methods. The results are indicated as a mean \pm SEM of five mice in each group. C: The lungs were taken from B6, J\(\alpha\)18\(^{-/-}\), and CD1d\(^{-/-}\) mice with or without an intratracheal injection of bleomycin (2 mg/kg) on day 21, and the hydroxyproline content was determined. The increased hydroxyproline contents in the lungs of experimental groups were presented as percentage. The results are indicated as a mean \pm SEM of nine mice in each group. D and E: B6, J\(\alpha\)18\(^{-/-}\), and CD1d\(^{-/-}\) mice were administrated with a high
dose of bleomycin (3 mg/kg), and the body weight and survival rate were measured. The results are indicated as a mean \pm SEM of six mice in each group. The results from a representative of three repeated experiments are shown. Original magnifications, \(x\)200 (A).
concentrations of IFN-\(\gamma\) in the BAL fluid and lung tissues. In contrast, the amounts of TGF-\(\beta\)1 in the lung tissues and BAL fluids from CD1d\(^{-/-}\) mice were higher than those of wild-type B6 mice in the bleomycin-induced pulmonary fibrosis model (Figure 5, A and B). The subsequent experiment was designed to examine whether or not the production of these cytokines in the lung is restored by the reconstitution of NKT cells in CD1d\(^{-/-}\) mice treated with bleomycin. The TGF-\(\beta\)1 transcript in the lung was restored in CD1d\(^{-/-}\) mice and was comparable to that of wild-type B6 by the adoptive transfer of NKT cells during bleomycin-induced pulmonary fibrosis (Figure 4, B and C). These findings suggest that the significant production of these cytokines produced during pulmonary fibrosis was similar in wild-type B6 and CD1d\(^{-/-}\) mice (data for IL-13 not shown). To establish a functional link between the TGF-\(\beta\)1 activity and pulmonary fibrosis, a blocking anti-TGF-\(\beta\)1 mAb was injected intravenously into CD1d\(^{-/-}\) mice, as described in Materials and Methods. The TGF-\(\beta\)1 blockade in CD1d\(^{-/-}\) mice induced a significant decrease in the bleomycin-induced pulmonary fibrosis when compared with the control antibody (Figure 6A). These results indicate that the TGF-\(\beta\)1 blockade in CD1d\(^{-/-}\) mice results in a similar phenotype that is found in normal B6 mice with bleomycin-induced pulmonary fibrosis.
induced pulmonary fibrosis. These findings show that the regulatory effects of NKT cells on pulmonary fibrosis depended on the amount of TGF-β1 produced in the lung.

The Production of TGF-β1 in the Bleomycin-Treated Lung Is Regulated by NKT Cells Producing IFN-γ

To determine how NKT cells regulated the production of TGF-β1 in the bleomycin-treated lung, the BAL cells were taken from wild-type B6 mice injected with bleomycin, and cultured with ConA. On activation of NKT cells using α-GalCer, the production of TGF-β1 was suppressed and restored by blocking the interaction between CD1d and TCR of NKT cells using anti-CD1d mAb (Figure 6B). This indicates that activation of NKT cells specifically suppressed the production of TGF-β1. It was reported that NKT cells rapidly secret a large amount of IL-4, IFN-γ, and IL-13, and enhance IL-10 production on activation by α-GalCer \textit{in vitro}. \textsuperscript{22,32–35} Therefore, it was proposed that these cytokines produced by NKT cells play an important role in regulating the immune responses by NKT cells. This study next examined which cytokines were involved in the suppression on TGF-β1 production by NKT cells. IL-4, IL-10, IL-13, and IFN-γ were neutralized using the mAbs during the activation of BAL cells and NKT cells in \textit{in vitro} culture. Among these blocking mAbs tested, mAbs against IFN-γ partially restored production of TGF-β1 whereas mAbs against IL-4, IL-10, and IL-13 as well as control IgG could not affect production of TGF-β1 in culture (Figure 6B). This suggests that NKT cells produce IFN-γ, which is involved in the suppression of TGF-β1 produced by BAL cells. To address this hypothesis, the amount of IFN-γ produced by BAL cells was measured after treating BAL cells with α-GalCer. α-GalCer induced IFN-γ production by the BAL cells in a dose-dependent manner, which was
the development of pulmonary fibrosis and the suppression of TGF-$\beta_1$ production in the lung. As observed in the in vitro assay, IFN-$\gamma$ secreted from NKT cells down-regulated TGF-$\beta_1$ transcript level during the development of pulmonary fibrosis. Altogether, these results demonstrated that IFN-$\gamma$ produced by NKT cells played important roles in attenuating bleomycin-induced pulmonary fibrosis by suppressing TGF-$\beta_1$ production in the lung.

Discussion

During the development of bleomycin-induced pulmonary fibrosis, NKT cells, recognized by $\alpha$-GalCer/CD1d dimer, infiltrated the lung tissue of B6 mice whereas NKT cells were not detected in CD1d$^{-/-}$ mice. Subset analysis for the BAL fluids revealed that NKT cells infiltrated the lung 5 to 9 days after administering the bleomycin. The deficiency of NKT cells in B6 mice aggravates the bleomycin-induced pulmonary fibrosis, as estimated by the hydroxyproline content and histological analysis. The aggravated pulmonary fibrosis to bleomycin in NKT cell-deficient mice was also associated with a higher degree of weight loss and mortality when compared with wild-type B6 mice (Figure 2, D and E). In contrary, Kimura and colleagues reported that mortality in wild-type B6 mice was ~50% by 28 days after administrating the bleomycin, which was similar to the 65% mortality in Jax$^{18/-}$ mice. In their experiments, they administrated high bleomycin concentration (0.5 mg per mouse) into the mice for pulmonary fibrosis, which is in stark contrast to the dose used in our experiments (0.04 or 0.06 mg per mouse). It appears that the large amounts of bleomycin used in their experiments were inappropriate for evaluating the differences of mortality between wild-type B6 and Jax$^{18/-}$ mice in the bleomycin-induced pulmonary fibrosis model because high bleomycin concentrations used in their experiments caused severe acute injury to the lung tissues that even wild-type B6 mice could not overcome. Therefore, it is likely that a large discrepancy in the dose of the bleomycin administrated affects the mortality rate of the mice in two independent experiments. In addition, some NKT cell-deficient mice died before day 7 of intratracheal bleomycin injection, which appears to be an early time for the induction of fibrosis in the lung tissues. It is possible that the poor clinical outcome of the NKT cell-deficient mice in the early phase may be due to a bleomycin-induced acute lung injury rather than to pulmonary fibrosis. Therefore, it is likely that both bleomycin-induced acute lung injury and pulmonary fibrosis may contribute to the poor overall survival rate of CD1d$^{-/-}$ and Jax$^{18/-}$ mice in the bleomycin-induced pulmonary fibrosis.
fibrosis model. The critical role of NKT cells during the development of bleomycin-induced pulmonary fibrosis was further substantiated by the fact that adoptive transfer of NKT cells from normal B6 or RAG−/− Va14+ Vβ8.2+ mice restored the impaired fibrosis in CD1d−/− mice. Altogether, these findings suggest that NKT cells play important roles in the development of bleomycin-induced pulmonary fibrosis.

Among the cytokines tested, TGF-β1 production was prominent in the lungs of CD1d−/− mice compared with that in control B6 mice, and the amount of TGF-β1 produced in the lung closely correlated with the bleomycin-induced pulmonary fibrosis in CD1d−/− mice. Moreover, the adoptive transfer of either NKT cells from B6 or splenocytes from RAG−/− Va14+ Vβ8.2+ mice into CD1d−/− mice decreased the level of TGF-β1 transcription in the lung. The TGF-β1 blockade in CD1d−/− mice resulted in reducing the level of pulmonary fibrosis and increasing the survival rate in the bleomycin-induced pulmonary fibrosis. Taken together, these findings suggest that TGF-β1 is involved in the bleomycin-induced pulmonary fibrosis of CD1d−/− mice, which is tightly regulated by NKT cells in the lung.

In animal models of pulmonary fibrosis, the TGF-β1 mRNA and protein levels are increased in the lung tissues. TGF-β1 is both mitogenic and chemotactic for the fibroblasts, monocytes, and macrophages, resulting in the promoting accumulation of the extracellular matrix proteins by increasing their synthesis while inhibiting matrix degradation. Although the precise mechanism of the pulmonary fibrosis remains enigmatic, TGF-β1 signaling appears to be one of the important mediators of pulmonary fibrosis in both animals and humans. Therefore, regulating the endogenous TGF-β1 production and signaling in the pulmonary fibrosis has been regarded as the main issue for therapeutic purposes. Among the cytokine network, IFN-γ has been shown to suppress TGF-β1 expression and directly suppress fibroblast collagen production in vitro. Recently, Jiang and colleagues demonstrated that IFN-γ produced by NK cells in the lungs regulate bleomycin-induced pulmonary fibrosis in CXCR3-deficient mice, suggesting that innate burst of IFN-γ production in the lungs may play important roles for attenuating pulmonary fibrosis in vivo system.

In this study it was shown that a NKT cell/IFN-γ regulatory circuit represses TGF-β1 production in bleomycin-induced pulmonary fibrosis. Segal and colleagues reported that the majority (54%) of the IFN-γ-producing lymphocytes during the development of bleomycin-induced pulmonary fibrosis in mice were CD4+, 27%, 11%, and 5% were NK1.1+, CD8+, and B220+, respectively. Although the proportion of NKT cells in the IFN-γ-producing lymphocytes was not clarified in this study, NKT cells were considered to be included in NK1.1+ cells or CD4+ T cells. These results suggested that NKT cells might be a subset of T cells that produce IFN-γ and suppress TGF-β1 production during the development of bleomycin-induced pulmonary fibrosis. However, Kimura and colleagues demonstrated that the injection of α-GalCer into B6 mice induced the production of IFN-γ, which attenuated pulmonary fibrosis. IFN-γ induced by injection of α-GalCer was mainly produced by conventional T cells and NK cells rather than NKT cells, which suggests that NKT cells participate in the activation of conventional T cells rather than be a major source of IFN-γ in attenuating the bleomycin-induced pulmonary fibrosis by α-GalCer treatment. In contrast, our results revealed that the activation status, determined by expression levels of CD25 or CD69 on cell surface, of conventional NK1.1− TCRβ+ T cells in BAL fluid from B6 mice administered bleomycin was similar to that in CD1d−/− mice (Figure 1E). Moreover, the intratracheal administration of bleomycin induced pulmonary fibrosis in RAG−/− Va14+ Vβ8.2+ mice, which contain iNKT cells and NK cells in the absence of conventional T and B cells, suggesting that conventional T cells do not play essential roles in the development of bleomycin-induced pulmonary fibrosis. Overall, it is unlikely that NKT cells contribute to the activation of conventional T cells during the development of pulmonary fibrosis. In the present study, our results demonstrated that NKT cells produced IFN-γ during bleomycin-induced pulmonary fibrosis, and IFN-γ secreted by NKT cells contributed to the attenuation of pulmonary fibrosis. Several lines of evidence in this study suggest the critical roles of IFN-γ secreted by NKT cells on the anti-fibrotic effects in the bleomycin-induced pulmonary fibrosis. First, the level of IFN-γ transcription was lower in the lungs of CD1d−/− than in the B6 mice in the bleomycin-induced pulmonary fibrosis. Second, α-GalCer induced the production of IFN-γ by BAL cells in a dose-dependent manner in the in vitro culture experiment. Third, the adoptive transfer of NKT cells into CD1d−/− mice restored the amount of hydroxyproline and the transcriptional level of IFN-γ, and reduced the levels of TGF-β1 transcription in the lung, which were comparable to those of B6 mice in the bleomycin-induced pulmonary fibrosis model. In contrast, the adoptive transfer of IFN-γ-deficient NKT cells did not restore the amount of hydroxyproline and level of TGF-β1 transcription. Fourth, a blockade of IFN-γ using neutralizing mAb aggravated the pulmonary fibrosis in RAG−/− Va14+ Vβ8.2+ mice as well as in B6 and CD1d−/− mice, suggesting that conventional T cells are not the major source of IFN-γ in attenuating pulmonary fibrosis by NKT cells. Taken together, these findings indicate that NKT cells are a major subset of immune cells producing IFN-γ in the bleomycin-induced pulmonary fibrosis model. In vitro experiments demonstrated that the priming condition favoring a high level of IL-4 production and/or a low level of IFN-γ production greatly enhanced the TGF-β1 production in the secondary cultures. It appears that IFN-γ inhibits the differentiation of TGF-β1-producing CD4+ T cells. IFN-γ also inhibits the TGFβ-induced phosphorylation of Smad3 and the activation of the TGFβ-responsive genes. Thus, it has been proposed that IFN-γ contributes to the anti-fibrotic effects on pulmonary fibrosis by suppressing TGF-β1 production in the lung. Consistent with this hypothesis, IFN-γ has been shown to have anti-fibrotic effects by directly suppressing fibroblast type I and III collagen synthesis as well as TGF-β1 production during pulmonary fibrosis. Therefore, NKT cells contribute to the anti-fibrotic effects on
bleomycin-induced pulmonary fibrosis by producing IFN-γ, which suppresses the production and signaling of TGF-β1 in the lung.

Kinetic analysis for IFN-γ production in the BAL and lung tissues of B6 mice revealed two time points (days 1 and 7) showing peak in amounts of IFN-γ. In contrast, the amounts of IFN-γ in the BAL and lung tissues of CD1d−/−/H11002 mice were low at day 7 whereas they were high on day 1 like in the B6 mice. This suggests that NK cells might contribute to the production of large amounts of IFN-γ in the BAL and lung tissues approximately at day 7. Kinetic analysis of the NKT cell influx in the lungs of the wild-type B6 mice showed that the influx of NKT cells peaked at approximately day 7 and a small number of NKT cells infiltrated lung tissues at day 1 in the bleomycin-induced pulmonary fibrosis model. It is possible that the infiltrated NKT cells in the lung regulate bleomycin-induced pulmonary fibrosis by producing large amounts of IFN-γ at approximately day 7. However, it is also possible that NK cells rather than NKT cells produce IFN-γ in the BAL and lung tissues during the early stage of injury because a small number of NKT cells infiltrated the lung tissues and the concentration of IFN-γ in the lung of CD1d−/−/H11002 mice were similar to those in B6 mice in early phase of bleomycin-induced pulmonary fibrosis. Therefore, it is likely that NK cells contribute to producing large amounts of IFN-γ during the early phase (at day 1) and that NK cells regulate collagen synthesis during the later phase (approximately day 7) in the bleomycin-induced pulmonary model by producing IFN-γ in the lung tissues. In a human system, therapeutic approaches using IFN-γ has been attempted on the patients with idiopathic pulmonary fibrosis. In a human model, the influx of NK cells rather than NKT cells produce IFN-γ in the lung tissues.

In summary, during the bleomycin-induced pulmonary fibrosis, NK cells infiltrate the lung parenchyma and secrete a large amount of IFN-γ, which suppresses TGF-β1 production in the lung. Therefore, NK cells attenuate bleomycin-induced pulmonary fibrosis by producing IFN-γ in the lung tissues.

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