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*J Immunol* 2004; 172:5782-5789; doi: 10.4049/jimmunol.172.9.5782

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Treatment with α-Galactosylceramide Attenuates the Development of Bleomycin-Induced Pulmonary Fibrosis

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Pulmonary fibrosis is an end-stage disorder for which efficacious therapeutic options are not readily available. Although its pathogenesis is poorly understood, pulmonary fibrosis occurs as a result of various inflammations. NKT cells modulate inflammation because of their ability to produce large amounts of cytokines by stimulation with their glycolipid ligand. In the present study, we investigated the effects of α-galactosylceramide (α-GalCer), a selective NKT cell ligand, on the development of bleomycin-induced pulmonary fibrosis. Treatment of mice with α-GalCer prolonged their survival under bleomycin administration by attenuating the development of pulmonary fibrosis. The protective effects of α-GalCer were associated with an increase in the pulmonary level of IFN-γ and a decrease in the pulmonary level of fibrogenic cytokines such as TGF-β and connective tissue growth factor. The initial pulmonary inflammation caused by bleomycin was also attenuated by α-GalCer with the reduction of the macrophage inflammatory protein-2 level. The protective effects of α-GalCer were markedly reduced in mice lacking NKT cells or as a result of treatment with anti-IFN-γ Ab. These results suggest that α-GalCer suppresses bleomycin-induced acute pulmonary inflammation and thus attenuates the development of pulmonary fibrosis possibly by regulating several cytokine levels.


NKT cells, typically defined as NK1.1⁺αβTCR⁺ cells in mice, are a novel lymphoid lineage distinct from T, B, or NK cells in the mouse immune system. Several studies have demonstrated that NKT cells participate in several immune and inflammatory responses because of their remarkable capacity to produce immunoregulatory cytokines such as IFN-γ and IL-4 after stimulation with either anti-CD3 (7, 8) or their glycolipid ligand (9, 10).

α-Galactosylceramide (α-GalCer), a synthetic glycolipid originally isolated from marine sponges, is specifically recognized in a CD1d-restricted manner by Vα14⁺ NKT cells. Exogenously administered α-GalCer induces several immune responses including antitumor activity (11), granuloma formation (12), and host resistance to Toxoplasma gondii and Cryptococcus neoformans infections (13) by the activation of NKT cells to produce a large amount of IFN-γ. Moreover, IFN-γ is capable of activating NK cells and T cells to produce IFN-γ in a direct manner or by producing IL-12 from various APCs (14–16). It is therefore likely that α-GalCer influences the natural history of the development of pulmonary fibrosis.

Bleomycin is an antitumor drug often used as an inducing agent in models of pulmonary fibrosis. Intratracheal administration of bleomycin in mice induces acute pulmonary inflammation followed by varying degrees of intraalveolar and interstitial fibrosis (17–19). This fibrotic process resembles that in human pulmonary fibrosis (18, 20). In the present study, therefore, we investigated the effects of α-GalCer on the development of pulmonary fibrosis using this fibrosis model. The effects of α-GalCer were further compared between wild-type mice and NKT cell-deficient mice to evaluate the contribution of NKT cells.

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Received for publication April 28, 2003. Accepted for publication February 24, 2004.

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0022-1767/04/$02.00

Abbreviations used in this paper: α-GalCer, α-galactosylceramide; BAL, bronchoalveolar lavage; CTGF, connective tissue growth factor; MIP-2, macrophage inflammatory protein-2.
the best for evaluating both the degree of fibrosis and the survival rate. 

Histopathologic assessment
The lungs were removed 28 days after bleomycin or saline administration. Following fixation, the lungs were embedded in paraffin. The sections were then stained with Masson’s trichrome stain. Histopathologic evaluation of pulmonary fibrosis was performed using a previously described scoring method (21). Briefly, the grade of lung fibrosis was scored on a scale of 0 to 8 using the following criteria: grade 0, normal lung; grade 1 to 2, minimal fibrous thickening of alveolar or bronchiolar wall; grade 3 to 4, moderate thickening of walls without obvious damage to lung architecture; grade 5 to 6, increased fibrosis with definite damage to lung structure; grade 7, severe fibrosis with destruction of alveolar structure; grade 8, fibroproliferative lesions.

Materials and Methods

Mice
Wild-type C57BL/6 mice were obtained from Charles River Breeding Laboratories Japan (Kanagawa, Japan). NKT cell-deficient (Jo281−/−) mice were established by specific deletion of the Jo281 gene segment with homologous recombination and aggregation chimera techniques (9). All mice used in this study were 6–8 wk of age and were maintained in our animal facilities under specific pathogen-free conditions. The studies adhered to the National Institutes of Health guidelines for the experimental use of animals. All animal studies were approved by the Institutional Review Board.

Reagents
A synthetic form of α-GalCer, KRN7000, was kindly provided by Kirin Brewery (Gunma, Japan). The lyophilized form of α-GalCer was diluted in 1 ml of distilled water to make a stock solution. This stock solution was further diluted into an appropriate concentration with saline and used for the experiments. The vehicle control stock solution was also diluted with saline and used for all experiments.

Treatment of mice
Mice were i.p. injected with α-GalCer (10 µg/100 µl vehicle) or control vehicle. One day after the treatment with α-GalCer or vehicle, these mice were administered bleomycin (0.5 mg/100 µl saline; Calbiochem, San Diego, CA) or saline intratracheally. We preliminarily examined 0.1 mg, 0.5 mg, and 1 mg of bleomycin and concluded that 0.5 mg of bleomycin was the best for evaluating both the degree of fibrosis and the survival rate.

Histopathologic assessment
The lungs were removed 28 days after bleomycin or saline administration. Following fixation, the lungs were embedded in paraffin. The sections were then stained with Masson’s trichrome stain. Histopathologic evaluation of pulmonary fibrosis was performed using a previously described scoring method (21). Briefly, the grade of lung fibrosis was scored on a scale of 0 to 8 using the following criteria: grade 0, normal lung; grade 1 to 2, minimal fibrous thickening of alveolar or bronchiolar wall; grade 3 to 4, moderate thickening of walls without obvious damage to lung architecture; grade 5 to 6, increased fibrosis with definite damage to lung structure; grade 7, severe fibrosis with destruction of alveolar structure; grade 8, fibroproliferative lesions.

FIGURE 1. α-GalCer prolongs the survival of wild-type mice following bleomycin administration. Mice were divided into four groups. The control group (●) was administered saline 1 day after treatment with vehicle (n = 20). The Gal group (■) was administered saline 1 day after α-GalCer treatment (n = 20). The Bleo group (○) was administered bleomycin 1 day after treatment with vehicle (n = 20). The Gal+Bleo group (□) were administered bleomycin 1 day after α-GalCer treatment (n = 20). A, Survival of wild-type C57BL/6 mice. Differences between the Bleo group (○) and the Gal+Bleo group (□) were significant (p < 0.05). B, Survival of Jo281−/− mice of the same background. There were no significant differences between the Bleo (○) and Gal+Bleo groups (□).

FIGURE 2. α-GalCer attenuates the development of bleomycin-induced pulmonary fibrosis. Mice were divided into four groups as described in Fig. 1. A, Photomicrograph of lung tissues from wild-type mice 28 days after the administration of bleomycin or saline is shown. Dense fibrosis was observed in the lungs of the Bleo group, whereas the fibrosis was limited in the Gal+Bleo group. B, Photomicrograph of lung tissues from Jo281−/− mice 28 days after the administration of bleomycin or saline is shown. The degree of fibrosis was similar between the Bleo and Gal+Bleo groups. Masson’s trichrome stain with scale bar = 100 µm. C, Quantitative assessment of pulmonary fibrosis 28 days after the administration of bleomycin or saline using a scoring method as previously described (n = 4 in each group). * Differences between the Bleo and Gal+Bleo groups were significant in wild-type mice (p < 0.05), but not in Jo281−/− mice.
Concentration of macrophage inflammatory protein (MIP)-2 was determined in the supernatant of the first BAL 2 days after bleomycin or saline administration by ELISA (Endogen, Cambridge, MA). Concentration of IFN-γ was determined in the lung homogenates and serum by ELISA (Endogen, Cambridge, MA). A concentration of the active form of TGF-β was also determined by ELISA (R&D Systems, Minneapolis, MN). To obtain the lung homogenates, the lungs were removed and homogenized using Polytron homogenizer (KINETIMATICA, Lucerne, Switzerland). After centrifugation for 1 h at 100,000 × g, the supernatants were recovered.

PCR analysis

Seven days after bleomycin or saline administration, total RNA was extracted from the lung tissues, and RT-PCR was performed using an AccessQuick RT-PCR System (Promega, Madison, WI), according to the manufacturer’s instructions. The sequences of the PCR primers were: TGF-β, 5′-GCCCTGGACACCAACTATTGCT-3′ and 5′-GCCACAGTGGG-3′; connective tissue growth factor (CTGF), 5′-CTATGCAGCAGTGACCTG-3′ and 5′-CTCCGCTACATCTCCGTAC-3′; and GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTGTTGTA-3′. Semiquantitative analysis was performed using the NIH Image software program (Bethesda, MD).

Neutralization of IFN-γ

To neutralize IFN-γ, mAb to IFN-γ (IFN-mAb, 1 mg/kg; BD PharMingen) or PBS was injected into wild-type mice 6 h before intrastracheal bleomycin or saline administration. Twenty-eight days after bleomycin or saline administration, the mice were sacrificed and the lungs were removed for the analysis.

Statistics

Data were expressed as mean ± SEM. Comparisons of data among each experimental group were performed using ANOVA and Scheffe’s test. The survival curves were analyzed using the log-rank test. Values of p < 0.05 were considered to be statistically significant.

Results

α-GalCer prolongs survival after bleomycin administration in wild-type mice

We first examined whether treatment with α-GalCer protects mice against bleomycin-induced lung toxicity. Wild-type C57BL/6 mice were intratracheally administered bleomycin or saline 1 day after α-GalCer or vehicle treatment. Fifty percent of the mice in grade 7 to 8; severe distortion of structure and large fibrous areas. After the examination of 30 randomly chosen regions in each sample at a magnification of ×100, the mean score of all 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 (22).

Bronchoalveolar lavage (BAL)

Two days after bleomycin or saline administration, the lungs were lavaged with six sequential aliquots of 1 ml of PBS. The supernatant of the first BAL was used to analyze albumin concentration by means of its color reaction with bromphenol blue (Sigma-Aldrich, St. Louis, MO) at 630 nm. The remaining pooled BAL was centrifuged 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

After 2 days and 7 days of bleomycin or saline administration, the lungs were removed, minced, and incubated with RPMI 1640 containing 10% FCS and 25 U/ml collagenase (type 1, Sigma-Aldrich) at 37 °C for 90 min. The cells were then filtered through 20-μm nylon mesh. The cell suspensions were stained with anti-NK1.1, anti-CD3, anti-CD4, and anti-CD8 Abs (BD PharMingen, San Diego, CA). After staining, the cells were analyzed by flow cytometry using the FACSCaliber with CellQuest software (BD Biosciences, San Jose, CA).

Intracellular cytokine analysis

IFN-γ production in lung lymphocytes was determined by flow cytometric intracellular cytokine analysis as previously described (23). Briefly, cells were suspended at 106/ml in RPMI 1640 containing 10% FCS, incubated with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich) for 2 h, and then incubated with brefeldin A (10 μg/ml; Sigma-Aldrich) for 2 h at 37 °C. Cells were then washed in PBS and fixed with 2% formaldehyde in PBS for 15 min at room temperature. Fixed cells were washed in PBS supplemented with 0.5% BSA and 0.02% sodium azide (PBS/BSA/azide). For intracellular cytokine detection, cells were permeabilized with 0.5% saponin (Sigma-Aldrich) in PBS/BSA/azide, stained with anti-IFN-γ-PE Ab (BD PharMingen), and analyzed by three-color FACS analysis.

Measurement of cytokines

Concentration of macrophage inflammatory protein (MIP)-2 was determined in the supernatant of the first BAL 2 days after bleomycin or saline administration by ELISA (BioSource International, Camarillo, CA). Concentration of IFN-γ was determined in the lung homogenates and serum by ELISA (Endogen, Cambridge, MA). A concentration of the active form of TGF-β was also determined by ELISA (R&D Systems, Minneapolis, MN). To obtain the lung homogenates, the lungs were removed and homogenized using Polytron homogenizer (KINETIMATICA, Lucerne, Switzerland). After centrifugation for 1 h at 100,000 × g, the supernatants were recovered.

PCR analysis

Seven days after bleomycin or saline administration, total RNA was extracted from the lung tissues, and RT-PCR was performed using an AccessQuick RT-PCR System (Promega, Madison, WI), according to the manufacturer’s instructions. The sequences of the PCR primers were: TGF-β, 5′-GCCCTGGACACCAACTATTGCT-3′ and 5′-CCACAGTGGG-3′; connective tissue growth factor (CTGF), 5′-CTATGCAGCAGTGACCTG-3′ and 5′-CTCCGCTACATCTCCGTAC-3′; and GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCTGTGTTGTA-3′. Semiquantitative analysis was performed using the NIH Image software program (Bethesda, MD).
FIGURE 4. α-GalCer increases lung IFN-γ level. A, The proportion of the lung lymphocyte subset in wild-type mice (upper) and Jα281−/− mice (lower) 2 days (left) and 7 days (right) after the administration of bleomycin or saline. Mice were divided into four groups as described in Fig. 1. Representative plots in each group are shown, and the proportion given for each group is the mean ± SEM of four experiments. §, Significantly different from corresponding saline-administered controls (p < 0.05); †, significantly different from corresponding vehicle-treated controls (p < 0.05).

B, Intracellular production of IFN-γ in NKT cells (upper), NK cells (middle), and T cells (lower) in lung lymphocytes 2 days (left) and 7 days (right) after the administration of bleomycin in both wild-type and Jα281−/− mice. Mice were divided into four groups as described in Fig. 1. C, The analysis of the T cell subset in IFN-γ-producing cells in the Gal + Bleo group of wild-type mice 7 days after the administration of bleomycin. D, The concentrations of IFN-γ in lung homogenates (left) and in serum (right) 7 days after the administration of bleomycin or saline (n = 8 in each group). *, Significant difference (p < 0.05) between the control and Gal groups; #, significant difference (p < 0.05) between the Bleo and Gal + Bleo groups.

α-GalCer attenuates the development of pulmonary fibrosis

We then assessed the protective effects of α-GalCer on the development of bleomycin-induced pulmonary fibrosis. Fig. 2A shows the lung histology 28 days after bleomycin or saline administration in wild-type mice. Masson’s trichrome stain revealed dense fibrosis with prominent collagen deposition 28 days after bleomycin administration (Bleo) in the vehicle-treated group (Fig. 2A). The degree of pulmonary fibrosis was markedly decreased in the α-GalCer-treated group (Gal + Bleo), compared with the vehicle-treated group (Fig. 2A). No abnormal alveolar architecture was observed after saline administration in either the vehicle-treated (control) or α-GalCer-treated (Gal) groups (Fig. 2A).

In Jα281−/− mice, α-GalCer treatment did not prevent the development of pulmonary fibrosis. Dense fibrosis was observed in both vehicle-treated and α-GalCer-treated (Bleo and Gal + Bleo) groups 28 days after bleomycin administration (Fig. 2B). No abnormal alveolar architecture was observed after saline administration in either the vehicle-treated (control) or α-GalCer-treated (Gal) groups (Fig. 2B).

We then assessed the degree of pulmonary fibrosis using a scoring method. In wild-type mice, the α-GalCer treatment significantly decreased the scores of fibrotic lesions 28 days after bleomycin administration when compared with the vehicle-treated group (Fig. 2C). In Jα281−/− mice, however, there was no significant difference in the scores between vehicle-treated and α-GalCer-treated groups 28 days after bleomycin administration (Fig. 2C).
We further assessed the degree of pulmonary fibrosis by measuring the lung hydroxyproline content. In wild-type mice, α-GalCer treatment significantly decreased the lung hydroxyproline content 28 days after bleomycin administration when compared with the vehicle-treated group (Fig. 2D). In Jα281−/− mice, however, there was no significant difference in the hydroxyproline content between vehicle-treated and α-GalCer-treated groups 28 days after bleomycin administration (Fig. 2D).

α-GalCer suppresses acute pulmonary inflammation

We next evaluated the effects of α-GalCer on initial pulmonary inflammation induced by bleomycin. We preliminarily determined that acute pulmonary inflammation indicated by BAL albumin content and neutrophil number peaked at 2 days after the administration of bleomycin or saline.

Significant increases in albumin concentration and in the number of neutrophils were observed in BAL fluid 2 days after bleomycin administration in both wild-type and Jα281−/− mice, compared with saline-administered controls (Fig. 3, A and D). In wild-type mice, BAL albumin concentration was significantly reduced by treatment with α-GalCer from the value of 224.6 ± 44.9 mg/ml in vehicle-treated controls 2 days after bleomycin administration (Fig. 3A). Treatment with α-GalCer also significantly decreased the number of neutrophils in BAL from the value of 5.4 ± 0.5 million in vehicle-treated controls at that time (Fig. 3D).

In Jα281−/− mice, however, there were no significant differences in BAL albumin concentration or neutrophil number between vehicle-treated and α-GalCer-treated groups 2 days after bleomycin administration (Fig. 3, A and D). Although the number of macrophages in BAL fluids increased 2 days after bleomycin administration in both wild-type and Jα281−/− mice, treatment with α-GalCer did not affect the number of macrophages in either type of mice (Fig. 3B). Few lymphocytes were observed at that time in both types of mice (Fig. 3C).

α-GalCer increases lung IFN-γ levels after bleomycin administration

To clarify the effect of α-GalCer on NKT cell activation in the lung, we examined the proportion of the lymphocyte subset and IFN-γ production by the lung lymphocytes 2 days and 7 days after the administration of bleomycin or saline, during acute pulmonary inflammation and fibrosis. The proportion of NKT cells, identified by NK1.1 and TCRβ, in lung lymphocytes was <1% in wild-type control mice on both day 2 and day 7 (Fig. 4A). Although lung NKT cells were increased significantly on day 2 by bleomycin administration, treatment with α-GalCer did not affect the proportion of lung NKT cells (Fig. 4A). NKT cells were not detected in the lungs of Jα281−/− mice (Fig. 4A). The proportion of T cells in lung lymphocytes was significantly elevated in both wild-type and Jα281−/− mice at day 2 and day 7 by bleomycin administration (Fig. 4A). Treatment with α-GalCer did not alter the proportion of lung T cells (Fig. 4A). Conversely, the proportion of NK cells was significantly increased at day 2 by treatment with α-GalCer in wild-type mice (Fig. 4A).

We then evaluated the intracellular production of IFN-γ in NKT, NK, and T cells in the lungs 2 days and 7 days after the administration of bleomycin or saline. At day 2, the proportion of IFN-γ-positive NKT cells in total NKT cells was <1% in each group, and the proportion was not different among the groups in wild-type mice (Fig. 4B, upper left). Similarly, the proportions of IFN-γ-producing NK and T cells among the total cells were both <3%, and there was no difference among the groups (Fig. 4B, middle and lower left). The proportion of IFN-γ-positive NK and T cells was not different between wild-type mice and Jα281−/− mice at that time (Fig. 4B, lower left). At day 7, the proportion of IFN-γ-producing NKT cells was not different among the groups in wild-type mice (Fig. 4B, upper right). In contrast, IFN-γ-positive T cells were significantly increased by α-GalCer treatment at day 7 in wild-type mice whereas no significant increases were observed in Jα281−/− mice (Fig. 4B, lower right). The proportion of IFN-γ-positive NK cells did not differ among the groups at that time (Fig. 4B, middle right).

The T cell subset was then analyzed in IFN-γ-producing T cells from Jα281−/− mice 7 days after bleomycin administration. Among IFN-γ-positive T cells, 72.2 ± 2.7% were CD4-positive and 18.5 ± 1.6% were CD8-positive (Fig. 4C).

We further assessed the concentration of IFN-γ in lung homogenates 7 days after bleomycin administration. α-GalCer treatment significantly increased the concentration of IFN-γ in wild-type mice at that time (Fig. 4D, left). In Jα281−/− mice, α-GalCer did not alter the concentration of IFN-γ in lung homogenates (Fig. 4D, left). Serum IFN-γ levels were low, and there were no significant differences among the wild-type mice and Jα281−/− mice (Fig. 4D, right).

α-GalCer reduces MIP-2 and fibrogenic cytokine levels

To clarify the molecular mechanism of α-GalCer for protection against bleomycin-induced acute pulmonary inflammation, the
concentration of MIP-2, a potent neutrophil chemoattractant, was assessed in BAL fluids 2 days after the administration of bleomycin or saline. In both wild-type and Jα281−/− mice, the concentration of MIP-2 was significantly elevated 2 days after bleomycin administration (Fig. 5A). In wild-type mice, treatment with α-GalCer significantly reduced the MIP-2 concentration after bleomycin administration (Fig. 5A). However, in Jα281−/− mice, treatment with α-GalCer did not reduce the MIP-2 level at that time (Fig. 5A).

To determine the effects of α-GalCer on the expression of fibrogenic cytokines, we examined the expression of TGF-β and CTGF in the lungs 7 days after the administration of bleomycin or saline. Expression of both TGF-β and CTGF mRNAs was significantly up-regulated after bleomycin administration in the lungs of both wild-type and Jα281−/− mice (Fig. 5D). α-GalCer treatment significantly reduced the concentration of the TGF-β active form in the lungs of wild-type mice but not in Jα281−/− mice (Fig. 5D).

We further assessed the concentration of the active form of TGF-β in lung homogenate 7 days after the administration of bleomycin or saline. As in the expression of TGF-β mRNA, the protein level of the TGF-β active form was significantly elevated after bleomycin administration in the lungs of both wild-type and Jα281−/− mice (Fig. 5D). α-GalCer treatment significantly reduced the concentration of the TGF-β active form in the lungs of wild-type mice but not in Jα281−/− mice (Fig. 5D).

Anti-IFN-γ Ab abrogates the effects of α-GalCer
To determine the role of IFN-γ in α-GalCer-mediated protection against bleomycin-induced pulmonary fibrosis, IFN-mAb was administered to wild-type mice. IFN-mAb treatment abrogated the protective effects of α-GalCer against bleomycin. IFN-mAb significantly reduced the survival rate of the α-GalCer-treated group after bleomycin administration to the level of the vehicle-treated group (Fig. 6A). IFN-mAb did not affect the survival rate of the vehicle-treated group after bleomycin administration (Fig. 6A).

Lung histology shows that IFN-mAb treatment aggravated pulmonary fibrosis in the α-GalCer-treated group 28 days after bleomycin administration (Fig. 6B). Then numerical scores of the fibrotic lesions and lung hydroxyproline content also increased significantly in the α-GalCer-treated group 28 days after bleomycin administration by IFN-mAb treatment (Fig. 6, C and D).

The reduction of TGF-β and CTGF mRNA expression by α-GalCer was abrogated by treatment with IFN-mAb (Fig. 6E).

Discussion
The present study demonstrated that treatment of C57BL/6 mice with α-GalCer attenuated the development of pulmonary fibrosis and prolonged survival following bleomycin administration. It has been established that α-GalCer is a selective NKT cell ligand that binds to CD1d and strongly stimulates NKT cell functions such as

FIGURE 6. Treatment with anti-IFN-γ Ab abrogated the protective effects of α-GalCer. A, Survival of wild-type mice after the administration of bleomycin or saline. Mice were divided into six groups. The Gal group (△) was treated with α-GalCer 1 day before and with PBS 6 h before intratracheal saline administration (n = 18). The mAb group (□) was treated with vehicle 1 day before and with anti-IFN-γ Ab (mAb) 6 h before intratracheal saline administration (n = 18). The Bleo group (○) was administered bleomycin 1 day after treatment with vehicle (n = 20). The Gal+Bleo group (△) was administered bleomycin 1 day after α-GalCer treatment (n = 20). The mAb+Bleo group (□) was treated with vehicle 1 day before and with mAb 6 h before intratracheal bleomycin administration (n = 18). The mAb+Gal+Bleo group (○) was treated with α-GalCer 1 day before and with mAb 6 h before intratracheal bleomycin administration (n = 18). Differences between the Gal+Bleo and mAb+Gal+Bleo groups were significant (p < 0.01). B, Photomicrograph of lung tissues in each group 28 days after the administration of bleomycin or saline. The degree of fibrosis was more severe in the lungs of the mAb+Gal+Bleo group than in the Gal+Bleo group. Masson’s trichrome stain, with scale bar, 100 μm. C, Quantitative assessment of pulmonary fibrosis 28 days after the administration of bleomycin or saline using a scoring method as previously described (n = 3 in each group). D, Lung hydroxyproline contents 14 days after the administration of bleomycin or saline (n = 4 in each group). E, Expression of TGF-β and CTGF in the lungs of wild-type mice 7 days after the bleomycin administration. **, Significant difference (p < 0.01) between the mAb+Gal+Bleo and Gal+Bleo groups.
cytokine production (9, 10). In the present study, the protective effects of α-GalCer were not observed in Jα281<sup>−/−</sup> mice. These findings suggest that activation of NKT cells is required for α-GalCer-mediated protection against the development of pulmonary fibrosis.

The mechanisms by which α-GalCer attenuates the development of pulmonary fibrosis are unclear. However, among NKT cell-activated cytokines, IFN-γ is known to have anti-fibrotic effects in experimental animals including bleomycin-induced pulmonary fibrosis model (4, 5). Our study demonstrated that α-GalCer treatment increased the pulmonary IFN-γ level in wild-type mice. In addition, neutralization of endogenously synthesized IFN-γ with a specific Ab abrogated the protective effects of α-GalCer in wild-type mice. These findings suggest that the protective effects of α-GalCer against the development of pulmonary fibrosis are, at least in part, mediated by the production of IFN-γ. Other NKT cell-induced host responses, including host resistance to T. gondii and C. neoformans infections (13), granuloma reaction caused by mycobacterium cell walls (12), and elimination of metastatic tumors (9, 24) are known to accompany the increase in IFN-γ level.

In the analysis of the lymphocyte subset harvested from the lungs, a significant increase in NKT cells was observed 2 days but not 7 days after bleomycin administration. Treatment with α-GalCer did not affect the number of NKT cells. These results suggest that NKT cells are recruited into the lung by stimulation with bleomycin in the early period of inflammation. However, the proportion of IFN-γ-producing NKT cells was low and did not differ between α-GalCer-treated and vehicle-treated mice, suggesting that NKT cells are not the major cell source of IFN-γ. T cells were also significantly increased in the lungs 2 days and 7 days after bleomycin administration. On day 7, the proportion of IFN-γ-producing T cells was significantly elevated in wild-type mice as a result of treatment with α-GalCer, and the elevation paralleled the lung IFN-γ level. These results suggest that α-GalCer causes the activation of T cells to produce IFN-γ. NKT cells may participate in the activation of T cells because no increase in the T cell production of IFN-γ was observed in Jα281<sup>−/−</sup> mice. Kawakami et al. (13) demonstrated that a large amount of IFN-γ was produced 7 days after α-GalCer treatment in mice infected with C. neoformans. No such production was noted by this treatment in CD4 knockout mice, and CD4<sup>+</sup> T cells were thus thought to be a major source of late-phase IFN-γ production induced by α-GalCer. Furthermore, in an in vitro experiment, IFN-γ synthesis by spleen cells obtained from α-GalCer-treated mice on day 7 was completely abrogated both in CD4 knockout mice and NKT-deficient mice (13). These results clearly indicated that CD4<sup>+</sup>T cells are a major source of late-phase IFN-γ production induced by α-GalCer. Consistently, a majority of IFN-γ-producing T cells were shown to be CD4<sup>+</sup>-positive in the present study.

The contribution of T cells to the pathogenesis of bleomycin-induced pulmonary fibrosis is still controversial. The accumulation of lymphocytes has been reported in bleomycin-induced pulmonary fibrosis (25, 26). Depletion of lymphocytes by anti-CD4 and anti-CD8 mAbs inhibited pulmonary fibrosis associated with bleomycin (27). Bleomycin-induced pulmonary inflammation and fibrosis were not observed in athymic nude mice (28). These studies 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 (29, 30). Although further examinations are required to clarify the contribution of T cells to the pathogenesis of fibrosis, the present study first showed that T cells may act as a negative regulator against the development of pulmonary fibrosis by producing anti-fibrotic cytokines through treatment with α-GalCer.

It was interesting that lung NK cells were elevated by the treatment with α-GalCer, whereas NKT and T cells were elevated by bleomycin administration in the present study. It has been demonstrated that NK cells were the producer of IFN-γ after α-GalCer treatment at the early phase of infection with C. neoformans through the activation of NKT cells (13). Because the proportion of IFN-γ-producing NK cells was low and did not differ between the α-GalCer-treated and vehicle-treated groups, NK cells may not directly contribute to the increase in lung IFN-γ level in this model. The role of NK cells in α-GalCer-mediated protection against pulmonary fibrosis remains to be elucidated.

The administration of bleomycin up-regulates a variety of cytokines, including fibrogenic cytokine. Among these cytokines, TGF-β has a role in the development of fibrosis because Abs against TGF-β markedly inhibit the development of bleomycin-induced pulmonary fibrosis in mice (31). We demonstrated in this study that the treatment with α-GalCer reduced the expression of TGF-β and CTGF. The reduction of these expressions by α-GalCer was abrogated by treatment with mAb to IFN-γ. These findings did not contradict the previous findings that treatment with IFN-γ markedly reduces the bleomycin-induced increase in TGF-β expression (4). CTGF is a fibrogenic cytokine known to be up-regulated in the lungs of bleomycin-treated mice (32). It was demonstrated that CTGF mRNA expression is regulated by TGF-β (33). Therefore, it is reasonable that CTGF expression as well as that of TGF-β was down-regulated after α-GalCer treatment. Taken together, these findings suggest that the reduction of the expression of fibrogenic cytokines might be one of the inhibitory mechanisms of α-GalCer against the development of pulmonary fibrosis.

Although IFN-γ shows anti-fibrotic effects in-vitro and animal studies, the potential of IFN-γ for treating human pulmonary fibrosis is considered to be limited in view of the recent failures in the IFN-γ treatment clinical trials in the U.S. Pulmonary fibrosis is thought to develop following the initial inflammatory responses in both human idiopathic pulmonary fibrosis and bleomycin-induced pulmonary fibrosis. Our observation revealed that α-GalCer significantly decreased acute pulmonary inflammation without an increase in IFN-γ production in lung lymphocytes, suggesting that α-GalCer-mediated inhibition of acute pulmonary inflammation occurs independently with the increase in IFN-γ. The mechanism of the anti-inflammatory effects of α-GalCer is unclear; however, we found that the concentration of MIP-2 in BAL fluids, which was elevated by bleomycin administration, was significantly reduced by treatment with α-GalCer. MIP-2 is a CXC chemokine and shows potent chemotactic activity for neutrophils (34). Regulation of neutrophil chemotactic factors is likely to be an additional effect of α-GalCer on the protection against acute pulmonary inflammation and the subsequent fibrosis. However, NKT cell-mediated regulation of neutrophilic inflammation is controversial because a recent study demonstrated that monocyte chemoattractant protein 1-dependent recruitment of Vα14-NKT cells induced early host protection against Streptococcus pneumoniae by promoting the trafficking of neutrophils to the site of infection (35).

We considered that the use of α-GalCer has an advantage over the systemic administration of anti-fibrotic cytokines such as IFN-γ for the treatment of pulmonary fibrosis for the following reasons: 1) treatment with α-GalCer, even via i.p. injection, increased lung IFN-γ levels but not the serum level, so that the systemic side effects of IFN-γ can be reduced; 2) effects in addition to the production of IFN-γ, such as the regulation of chemokines, are expected to be induced by α-GalCer. However, some problems.
have to be solved to use α-GalCer for the treatment of pulmonary fibrosis. Several studies have demonstrated that α-GalCer induces not only IFN-γ but also Th2 cytokines such as IL-4 and IL-13 under different conditions (36–38). We are currently investigating the balance of IFN-γ and IL-4 in the lungs using this fibrosis model with α-GalCer in various amounts and at various administration schedules to determine the adequate use of α-GalCer for the treatment of pulmonary fibrosis. The inoculation route of α-GalCer also should be examined. However, our preliminary examination showed that intratracheal administration of α-GalCer did not attenuate the development of pulmonary fibrosis by bleomycin (data not shown).

We demonstrated in the present study that treatment with α-GalCer attenuates the acute pulmonary inflammation and subsequent fibrosis induced by bleomycin. This synthetic glycolipid therefore may be a candidate agent for the treatment of acute lung injury and pulmonary fibrosis. Furthermore, bleomycin is an antitumor drug commonly used to treat various types of tumors (39). The most important side effect of bleomycin is pulmonary toxicity, including pulmonary fibrosis (19). The administration of α-GalCer to such patients might be useful for decreasing the side effects of bleomycin.

References


