

**ORIGINAL****Antifibrotic effects of CXCR4 antagonist in bleomycin-induced pulmonary fibrosis in mice**

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**Abstract :** Circulating fibrocytes had been reported to migrate into the injured lungs, and contribute to fibrogenesis via chemokine-chemokine receptor systems including CXCL12-CXCR4 axis. Here we hypothesized that blockade of CXCR4 might inhibit the migration of fibrocytes to the injured lungs and the subsequent pulmonary fibrosis. To explore the antifibrotic effects of blockade of CXCR4, we used a specific antagonist for CXCR4, AMD3100, in bleomycin-induced pulmonary fibrosis model in mice. Administration of AMD3100 significantly improved the loss of body weight of mice treated with bleomycin, and inhibited the fibrotic lesion in subpleural areas of the lungs. The quantitative analysis demonstrated that treatment with AMD3100 reduced the collagen content and fibrotic score (Aschcroft score) in the lungs. Although AMD3100 did not affect cell classification in bronchoalveolar lavage fluid on day 7, the percentage of lymphocytes was reduced by AMD3100 on day 14. AMD3100 directly inhibited the migration of human fibrocytes in response to CXCL12 *in vitro*, and reduced the trafficking of fibrocytes into the lungs treated with bleomycin *in vivo*. These results suggest that the blockade of CXCR4 might be useful strategy for therapy of patients with pulmonary fibrosis via inhibiting the migration of circulating fibrocytes. *J. Med. Invest.* 60 : 127-137, February, 2013

**Keywords :** pulmonary fibrosis, fibrocyte, CXCR4, CXCL12, AMD3100

**INTRODUCTION**

Idiopathic pulmonary fibrosis (IPF) is a progressive and lethal disease of the lungs characterized by the proliferation of fibroblasts and deposition of extracellular matrix (1-3). The recent progress in

the understanding of molecular pathogenesis in IPF is characterized as the identification of the novel origin of lung fibroblasts in addition to resident fibroblasts including bone-marrow derived circulating fibrocytes and fibroblasts derived from the epithelial-to-mesenchymal transition (EMT) (4-8). To control the novel subsets of lung fibroblasts might lead to the development of a useful therapeutic strategy for IPF.

A number of studies have demonstrated the potential for bone marrow (BM)-derived circulating fibrocytes to enter tissues following injury and contribute

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to wound healing and fibrosis (9-13). Fibrocytes constitute approximately 0.5% of the peripheral blood leukocyte population that shows an adherent spindle shape in culture (10). They express markers of haematopoietic cells (CD34), leukocytes (CD11b, CD13 and CD45) as well as fibroblast products (collagens I, III, and fibronectin) (9-13). They appear to be derived from the differentiation of CD14<sup>+</sup> peripheral blood mononuclear cells (9-11) and differentiate into fibroblasts/myofibroblasts under the control of TGF- $\beta$  (10-12). The number of BM-derived fibroblasts and fibrocytes recruited into the injured lung can reach up to 10% and 25% of fibroblasts (13). Furthermore the marked expansion of circulating fibrocyte pool in patients with interstitial pneumonia and the positive correlation between the abundance of fibroblastic foci and the amount of lung fibrocytes in patients with IPF were reported (14, 15). The mechanisms involved in the trafficking of fibrocytes have been reported to be several chemokines/chemokine receptors including CXCL12/CXCR4, CCL21/CCR7, CCL2/CCR2 and CCL3/CCR5 (4, 5, 16-18). In particular, CXCL12/CXCR4 axis is thought to be a main axis to regulate the migration of fibrocytes *in vitro* and *in vivo* (4, 5).

We therefore hypothesized whether blockade of CXCR4 might inhibit the recruitment of fibrocytes into injured lungs and the subsequent pulmonary fibrosis. In the present study, we demonstrated that CXCR4 antagonist AMD3100 reduced pulmonary fibrosis via inhibiting the migration of fibrocytes. These results suggest that CXCR4 antagonist could be a novel therapy for patients with pulmonary fibrosis.

## METHODS

### *Mice and Material*

Seven-week-old C57BL/6 female mice were purchased from Charles River Japan, Inc. (Yokohama, Japan). Mice were maintained in the animal facility of the University of Tokushima under specific pathogen free conditions according to the guidelines of our university (19). AMD3100 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Bleomycin was purchased from Nippon Kayaku Co. (Tokyo, Japan). Recombinant human CXCL12 was purchased from R&D systems, Inc. (Minneapolis, MN, USA). PE-conjugated anti-mouse CXCR4 antibody, PE-Cy5 conjugated anti-mouse CD45

antibody (BD Biosciences-Pharmingen, San Diego, California, USA), anti-collagen type I (Rockland, Gilbertsville, Pennsylvania, USA), BD Cytofix/CytoPerm Plus Kit (BD Biosciences-Pharmingen), Collagenase IV (Gibco 17104-019), DEOXYRIBONUCLEASE (DNase) I (CALBIOCHEM, Madison, WI, USA) was used in this study.

### *Bleomycin and AMD3100 treatment*

Mice were anesthetized with sodium pentobarbiturate, and osmotic minipumps (model 2001, Alza Pharmaceuticals, Palo Alto, CA, USA) containing 200  $\mu$ l of saline with or without 125 mg/kg of bleomycin (Nippon Kayaku Co., Tokyo, Japan) were implanted subcutaneously in the left suprascapular lesion through an incision at the base of the neck (19-21). Bleomycin was constantly infused from the minipumps over 7 days as described in the manufacturer's guidelines. This model showed the inflammation in the early phase (from days 0 to 14) and the fibrosis in the late phase (from days 14 to 28) (19). The fibrotic lesions were observed mainly in the subpleural area with patchy distribution. As a control experiment, minipumps containing saline only were also used. AMD3100 was also administered using osmotic minipump from day 0 to 28. Body weight of mice was measured every three to four days. Each experiment was performed in at least five mice per group.

### *Histopathology*

The left lungs were inflated with 0.5 ml of 10% buffered Formalin and fixed, followed by embedding in paraffin. Sections (3 to 4  $\mu$ m) were stained with hematoxylin and eosin, and then examined by light microscopy. For the quantitative histological analysis of fibrotic changes induced by bleomycin, a numerical fibrotic scale was used (Ashcroft score) (22). In order to avoid observer's bias, all histological specimens were randomly numbered and interpreted in a blind fashion by two pathologists. The severity of the fibrotic changes in each lung section was assessed as a mean score of severity from observed microscopic fields. Fifteen fields within each lung section were randomly selected and evaluated at a magnification of  $\times 100$ . Each field was assessed individually for the severity of fibrotic changes and given a score of 0 (normal) to 8 (total fibrosis). Briefly, the graded score from 0 to 8 was defined as follows: Grade 0 = normal lung; Grade 1 = minimal fibrous thickening of alveolar or bronchiolar walls; Grade 3 = moderate

thickening of the walls without obvious damage to the lung architecture ; Grade 5=increased fibrosis with definite damage to the lung structure and the formation of fibrous bands or small fibrous masses ; Grade 7=severe distortion of the lung structure and large fibrous areas ; Grade 8=total fibrous obliteration of the field. If there was any difficulty in deciding between two odd-numbered categories, the field was given the intervening even-numbered grade. The mean score of all evaluated fields was considered the fibrotic score. To further evaluate the fibrotic changes, Masson's-trichrome staining was performed.

#### *Collagen assay*

The total amount of collagen in the right lungs was determined using the Sircol Collagen Assay kit (Biocolor, Northern Ireland) according to the manufacturer's instructions (19, 21). The Sircol dye reagent contains Sirius Red, which is an anionic dye with sulfonic acid side chain groups. These groups react with the side chain groups of the basic amino acids present in collagen. The specific affinity of the dye for collagen under the assay conditions is due to the elongated dye molecules becoming aligned parallel with the long, rigid structure of native collagen molecules that have an intact triple helix organization. Briefly, the right lungs were harvested on day 28 after implantation of the minipump containing bleomycin and homogenized in 0.5 M acetic acid (50 volumes to wet lung weight) containing about 1 mg pepsin/10 mg tissue residue. Each sample was incubated for 24 hours at room temperature with stirring. After centrifugation, 100  $\mu$ l of each supernatant was assayed. One milliliter of Sircol dye reagent, which binds to collagen, was added to each sample, and the mixtures were mixed for 30 minutes. After centrifugation, the pellets were suspended in 1 ml of the alkaline reagent included in the kit and the absorbance at 540 nm was read using a spectrophotometer. Collagen standard solutions were utilized to construct a standard curve. Collagens contain about 14% hydroxyproline by weight, and the collagen content obtained with this method correlates well with hydroxyproline content according to the manufacturer's data, and was expressed as the collagen content of the right lung ( $\mu$ g/lung).

#### *Bronchoalveolar Lavage*

Bronchoalveolar lavage was performed five times with saline (1 ml) using a soft cannula (23). After

counting cell number of the bronchoalveolar lavage fluid, cells were cytospun onto glass slides and stained with Diff-Quick (Baxter, Miami, FL, USA) for cell classification.

#### *Purification of fibrocytes*

Human fibrocytes were isolated according to previously published methods (5). Peripheral blood mononuclear cells (PBMCs) were isolated from human peripheral blood by using Ficoll density centrifugation (24). PBMCs were then cultured in DMEM supplemented with 20% FBS for 7 days. Typically,  $5 \times 10^7$  PBMCs were plated into each fibronectin coated flask. The adherent cells were harvested by using 0.125% trypsin. We used immunomagnetic selection to deplete B lymphocytes (anti-CD19 ; Miltenyi Biotech, Auburn, CA, USA), T lymphocytes (anti-CD3 : Miltenyi Biotech), and monocytes/macrophages (anti-CD14 : Miltenyi Biotech) from our crude fibrocyte preparation. The trypsinized cells were stained with anti-CD19, CD3, CD14 Abs coupled to magnetic beads. Labeled cells were then depleted by positive selection columns using AutoMacs (Miltenyi Biotech). The negatively selected cells were collected and used as human fibrocytes.

Murine fibrocytes were isolated according to previously published methods (4). The lungs were harvested from saline or BLM-treated mice on day 7 and minced with razor blades. The minced lungs were treated with collagenase IV and DNase I to produce single cell suspensions and then cultured in fibronectin-coated 10 cm-dish for 7 days. Finally adherent cells were harvested and stained with PE-Cy5 labeled anti-mouse CD45 Ab. Subsequently, the cells were fixed in 10% neutral buffered formalin, permeabilized by 0.3% saponin and then stained with biotin-conjugated anti-mouse collagen-I Ab, followed by Streptavidin-FITC. The stained cells were analyzed by flow cytometry. The CD45<sup>+</sup>Collagen I<sup>+</sup> cells were counted as murine fibrocytes in this study.

#### *Flow cytometric analysis*

Cells isolated from human peripheral blood were stained with FITC-labeled anti-CD45 and CXCR4 antibodies. The stained cells were performed on a FACScan flow cytometer (BD Biosciences- Pharmingen, San Diego, California, USA) using Cellquest 3.2.1f1 software. Single cell suspensions from lung tissue of bleomycin-treated mice were initially stained with PE-Cy5 labeled CD45. Subsequently, the cells

were permeabilized using cytofix/cytoperm (BD Biosciences-Pharmingen) and were stained with anti-Collagen I Ab pre-labeled with Alexa488 by using Alexa488 labeling kit. Two-color analysis of the stained cells was performed on a FACScan flow cytometer.

#### Chemotaxis

Chemotaxis on cells at  $1 \times 10^5$  /well was performed in Boyden chambers to DMEM contained with 0.1% FBS or CXCL12 through collagen-coated 8  $\mu$ m filters. Migration was assessed by counting the number of cells in five high-power fields with a light microscope.

#### Statistical analysis

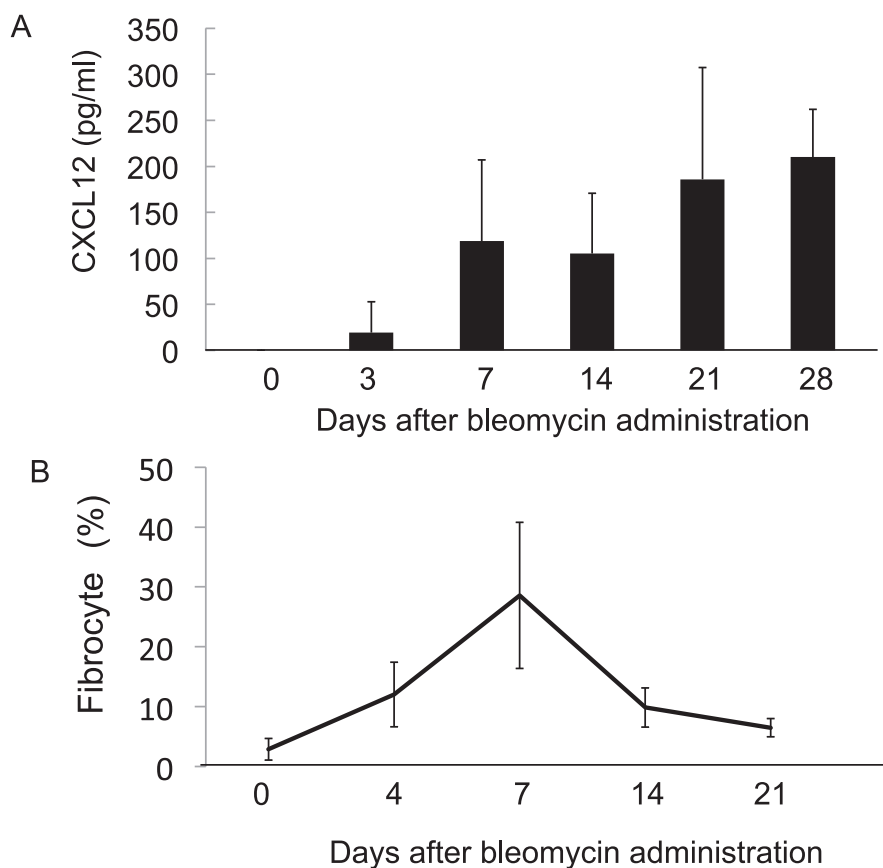
Comparisons among multiple groups were made using one-way ANOVA with Newman-Keuls post hoc correction (GraphPad Prism, version 4.0).

Differences were considered statistically significant if *p* values were less than 0.05.

## RESULTS

### *Time kinetics of CXCL12 and fibrocytes in the lungs treated with bleomycin*

First we analyzed the levels of CXCL12 in bronchoalveolar lavage fluid (BALF) of mice treated with bleomycin by ELISA. As shown in figure 1A, the levels of CXCL12 elevated from day 7 after bleomycin administration, and the high level continued until day 28. On the other hand, the number of fibrocytes was measured by flow cytometry using double staining of CD45 and collagen I. As shown in figure 1B, CD45<sup>+</sup>Collagen I<sup>+</sup> fibrocytes was increased from day 4 and reached the peak on day 7, and then gradually decreased until day 21.



**Figure 1** Time course of CXCL12 and fibrocytes in the lungs treated with bleomycin. Mice were treated with osmotic minipumps containing bleomycin. A: CXCL12 in bronchoalveolar lavage fluid (BALF) in mice treated with bleomycin. The BALF was harvested on days 3, 7, 14, 21 and 28. CXCL12 in BALF was measured by ELISA. Data are shown as the mean  $\pm$  SD of four mice. B: The percentage of fibrocytes in the lungs of mice treated with bleomycin. The lungs treated with bleomycin were harvested on days 0, 3, 7, 10, 14 and 21. The single cell suspensions were collected from the minced lungs. Then the cells were cultured on the dishes and the adherent cells were harvested. The double staining with anti-CD45 and collagen I antibodies were performed for flow cytometric analysis with adherent cells. The CD45<sup>+</sup>Collagen I<sup>+</sup> cells were counted as murine fibrocytes in this study. The Data are shown as the mean  $\pm$  SD of four mice.

*CXCR4 antagonist AMD3100 improves weight loss induced by bleomycin in mice*

Next, we examined the *in vivo* effects of a CXCR4 antagonist AMD3100 on weight loss induced by bleomycin in mice. The mice injected with bleomycin (125 mg/kg) showed a significant loss of body weight until day 14 as compared with those injected with saline, and weight loss gradually recovered from day 18. In contrast, mice treated with both bleomycin and AMD3100 showed a similar weight loss on day 14, but their weight quickly recovered from day 14 (Figure 2). On day 28, AMD3100 (3 mg/kg/day and 6 mg/kg/day) significantly improved the weight loss caused by bleomycin. Mice treated with 6 mg/kg/day of AMD3100 showed better tendency of recovery response as compared with mice treated with 3 mg/kg/day AMD3100, but there were no significant differences between two groups. On the other hand, mice treated with AMD3100 alone did not show any loss of body weight, indicating that 6 mg/kg/

day of AMD3100 was not toxic to mice.

*Administration of AMD3100 ameliorates bleomycin-induced lung fibrosis in mice*

The fibrotic change in the lung was evaluated by histological examination and measurement of total collagen content. As shown in Figure 3, administration of AMD3100 alone did not generate any changes in lung morphology, indicating that AMD3100 did not show any adverse effects on the lungs. However, when AMD3100 was administered daily to bleomycin-treated mice, a significant reduction of fibrosis in the lungs was observed (Figure 3). These antifibrotic effects of AMD3100 were also confirmed by histological examination using the fibrotic score as described in the Methods section (Figure 4A). The collagen assay demonstrated that treatment with AMD3100 dose-dependently reduced the production of total collagen in bleomycin-treated lungs (Figure 4B). More than 3 mg/kg/day of AMD3100 were effective in reducing the pulmonary fibrosis caused by bleomycin.

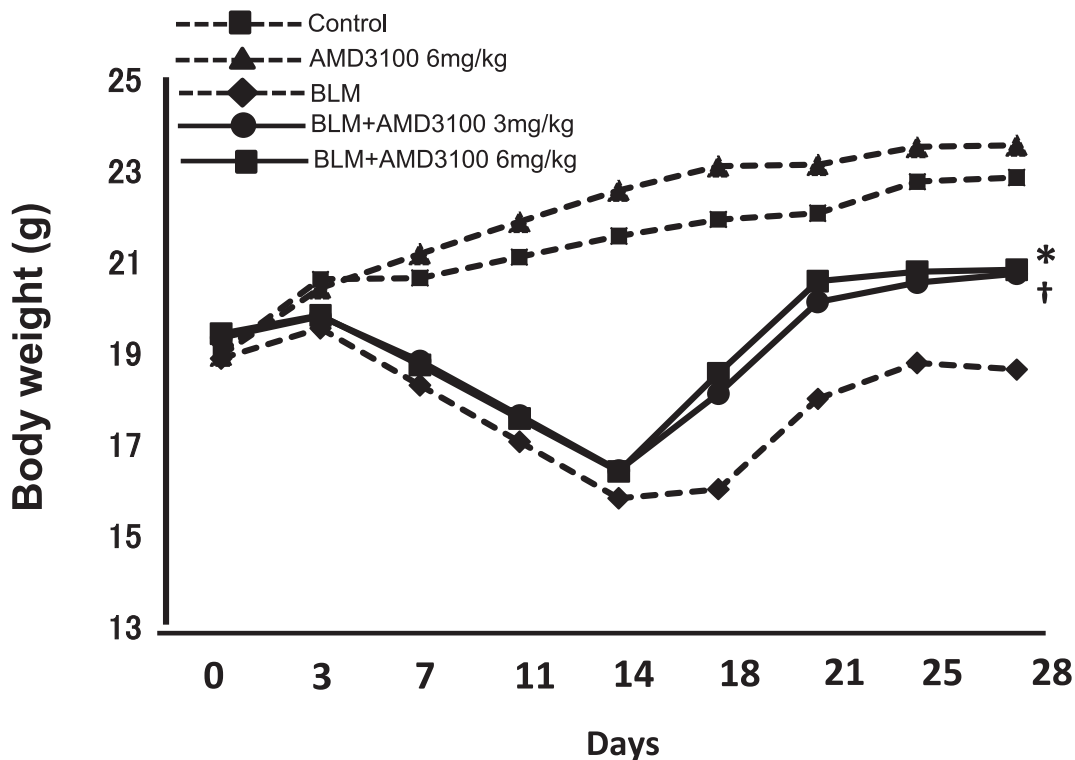
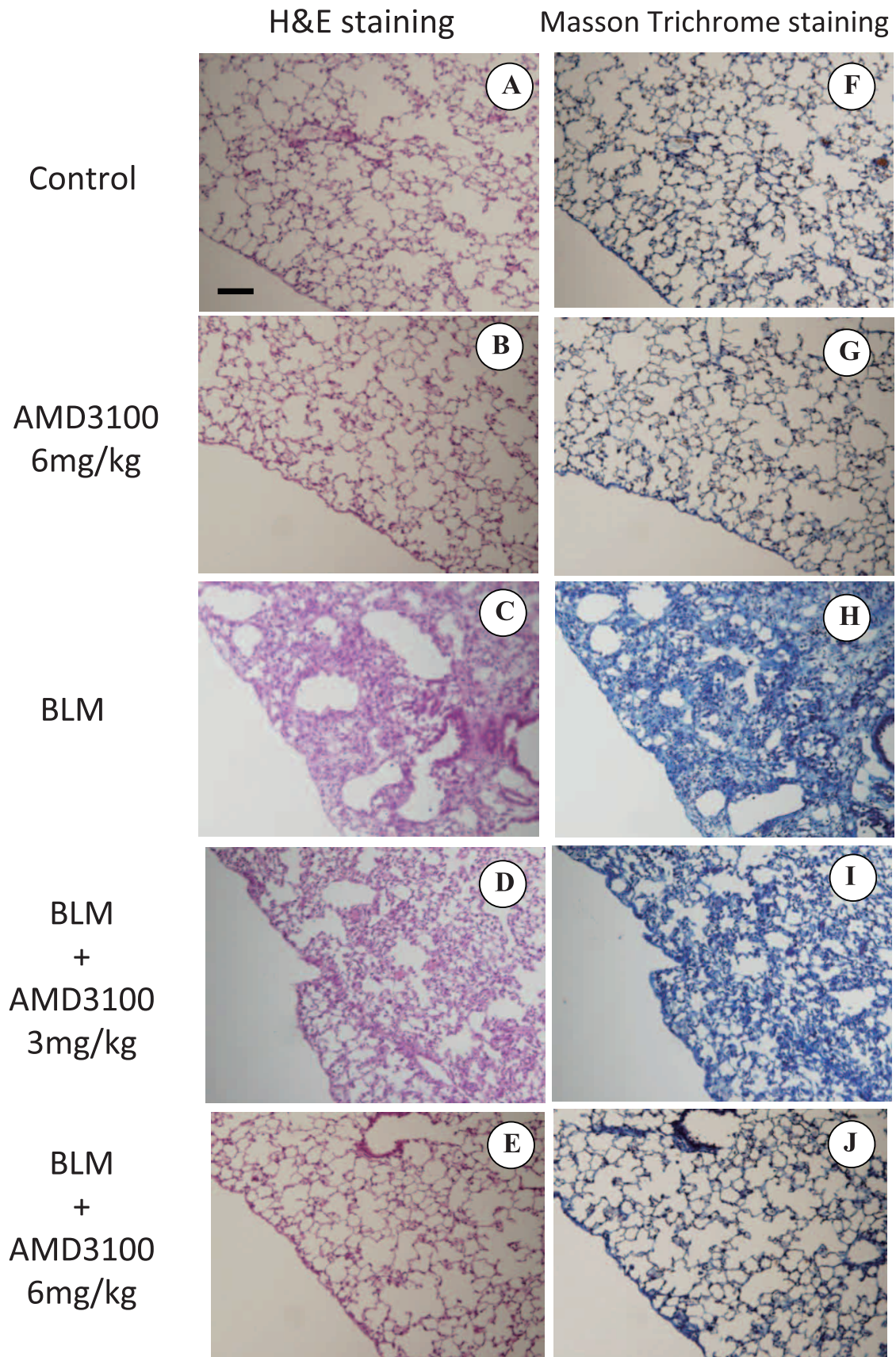
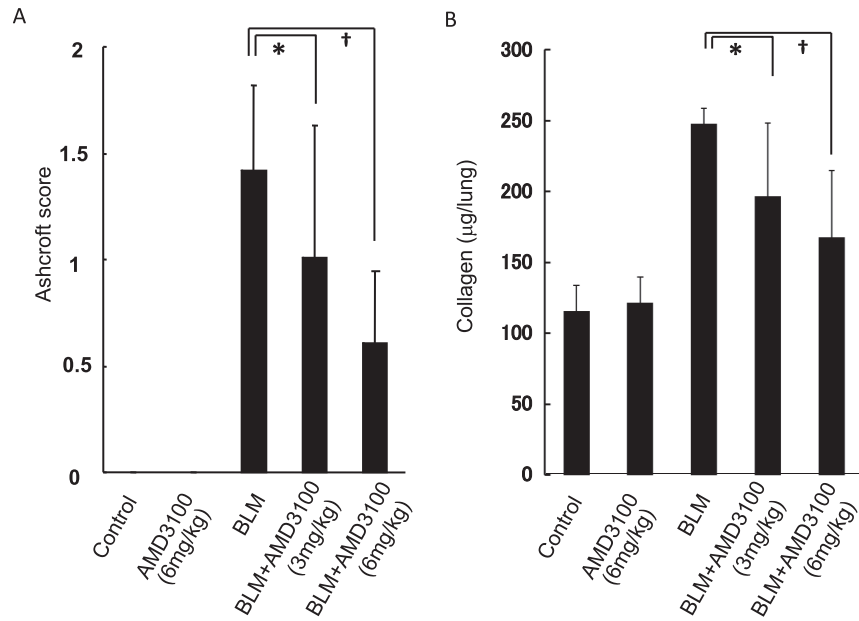


Figure 2 Effect of AMD3100 on weight loss of mice treated with bleomycin. Mice were treated with osmotic minipumps containing bleomycin (BLM). AMD3100 (3 mg/kg/day or 6 mg/kg/day) was given by using osmotic minipump. The change in body weight of mice treated with saline alone (n=5), AMD3100 (6 mg/kg/day) (n=5), Bleomycin alone (n=8), Bleomycin+AMD3100 (3 mg/kg/day) (n=8) or bleomycin+AMD3100 (6 mg/kg/day) (n=8) were examined. Similar results were obtained in two separate experiments. \* $p < 0.05$  versus bleomycin alone, † $p < 0.01$  versus bleomycin alone.



**Figure 3** Histological examination of the antifibrotic effects of AMD3100 in bleomycin-induced pulmonary fibrosis. Mice were treated with osmotic minipumps containing saline or bleomycin (BLM). AMD3100 (3 mg/kg/day or 6 mg/kg/day) was given subcutaneously by using osmotic minipump for the entire duration of the experiment. On day 28, mice were sacrificed and histological examination was performed by H&E staining (A-E) and Masson's-trichrome staining (F-J) (original magnification : x40). A, F : saline alone ; B, G : saline+AMD3100 (6 mg/kg/day) ; C, H : bleomycin alone ; D, I : bleomycin+AMD3100 (3 mg/kg/day) ; E, J : bleomycin+AMD3100 (6 mg/kg/day). Data are representative of three separate experiments. Bar=200  $\mu$ m.



**Figure 4** Quantitative examinations of the antifibrotic effects of AMD3100 on bleomycin-induced pulmonary fibrosis. Mice were treated with osmotic minipumps containing saline or bleomycin (BLM). AMD3100 (3 mg/kg/day or 6 mg/kg/day) was given by using osmotic minipump for the entire duration of the experiment. Mice were sacrificed on day 28. A : Evaluation of fibrotic change in the lung using numerical fibrotic score. Histological examination in the left lung was performed by H&E staining. The fibrotic score was determined by two pathologists as described in the Methods. Data are presented as mean  $\pm$  SD of all fields examined in each group of mice treated with saline alone (n=5), AMD3100 (6 mg/kg/day) (n=5), Bleomycin alone (n=8), Bleomycin+AMD3100 (3 mg/kg/day) (n=8) or bleomycin+AMD3100 (6 mg/kg/day) (n=8). B : Effects of AMD3100 on collagen deposition after treatment with bleomycin. Collagen content in the right lung was measured using a Sircol collagen kit. Data are presented as mean  $\pm$  SD of mice. Data are representative of two separate experiments. \* $p$ <0.05 versus bleomycin alone, † $p$ <0.001 versus bleomycin alone.

*Effect of AMD3100 on cell analysis of BALF of mice treated with bleomycin*

Next, we analyzed the cells in BALF to evaluate the effect of AMD3100 on the inflammatory responses induced by bleomycin. Administration of bleomycin elevated the number of inflammatory cells, including macrophages, lymphocytes and

neutrophils, on days 7, 14 and 28. Analysis of cell classification also showed that bleomycin treatment enhanced the percentage of lymphocytes and neutrophils on day 7, 14 and 28. Although administration of AMD3100 significantly reduced the percentage of lymphocytes in BALF on 14, AMD3100 did not affect the early inflammation in BALF on day 7 (Table 1).

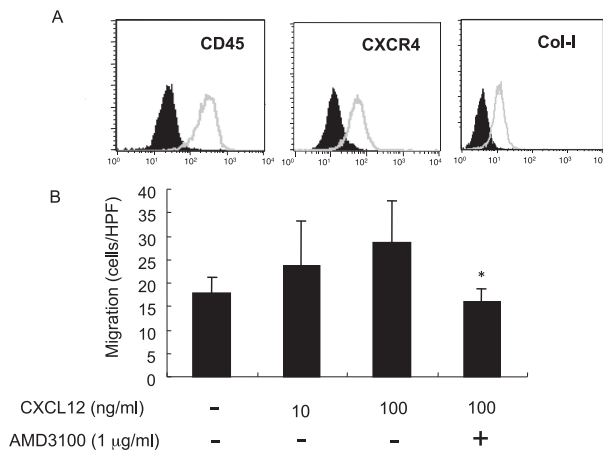
**Table 1** Effects of AMD3100 on cell classification of bronchoalveolar lavage fluid

Days after treatment	Total cells ( $\times 10^6$ )	Cell differentiation (%)		
		Macrophages	Lymphocytes	Neutrophils
Day 0	0.31 $\pm$ 0.15	95.6 $\pm$ 2.07	3.00 $\pm$ 1.73	1.40 $\pm$ 0.54
Day 7				
BLM	1.85 $\pm$ 0.26	73.3 $\pm$ 5.68	5.33 $\pm$ 1.52	21.3 $\pm$ 4.16
BLM+AMD3100	2.45 $\pm$ 0.35	67.3 $\pm$ 4.16	5.00 $\pm$ 1.00	15.2 $\pm$ 7.25
Day 14				
BLM	4.27 $\pm$ 1.53	67.6 $\pm$ 4.97	29.2 $\pm$ 4.65	3.20 $\pm$ 1.48
BLM+AMD3100	3.74 $\pm$ 1.51	85.0 $\pm$ 2.70	12.5 $\pm$ 2.38 *	2.50 $\pm$ 2.51
Day 28				
BLM	1.88 $\pm$ 0.57	79.2 $\pm$ 4.76	19.8 $\pm$ 4.43	1.20 $\pm$ 1.09
BLM+AMD3100	1.36 $\pm$ 0.89	79.5 $\pm$ 5.25	16.0 $\pm$ 4.76	4.25 $\pm$ 3.30

Mice were treated with mini-osmotic pump containing bleomycin (BLM) and AMD3100 (6 mg/kg/day). On day 0, 7, 14 and 28, bronchoalveolar lavage was performed as described in methods. Data are presented as mean  $\pm$  SD in the group of 6 mice. Similar results were obtained in three separate experiment. \* $p$ <0.01 v.s. percentage in BLM+AMD3100 group

### Effects of AMD3100 on the chemotaxis of human fibrocytes in response to CXCL12 *in vitro*

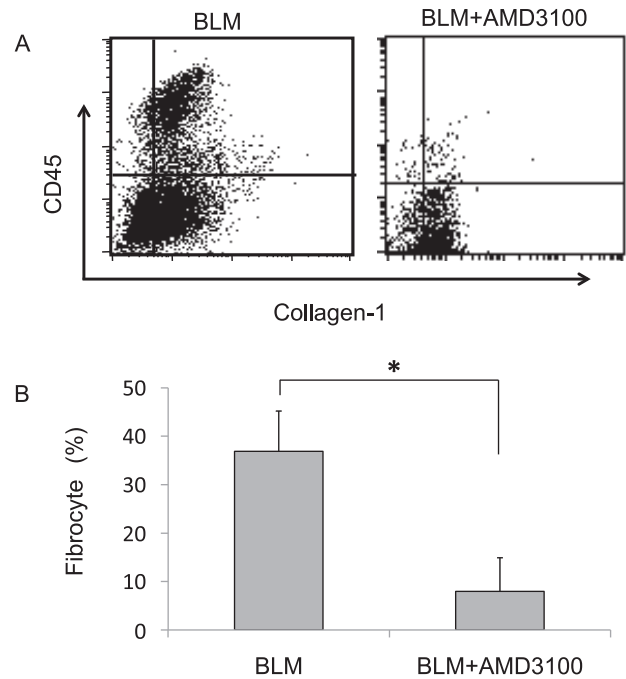
Next we examined whether AMD3100 inhibited the migration of human fibrocytes stimulated with CXCL12. Human fibrocytes were generated from peripheral blood mononuclear cells. These fibrocytes expressed CD45, CXCR4 and collagen I as previously described (figure 5A). Human fibrocytes migrated in response to CXCL12 in a dose-dependent manner (Figure 5B). Treatment with AMD3100 significantly inhibited the migration of human fibrocytes (Figure 5B).



**Figure 5** AMD3100 inhibits migration of human fibrocytes in response to CXCL12. A: Generation of human fibrocytes. Human fibrocytes were isolated from human peripheral blood mononuclear cells as described in the Methods. Cell surface markers of human fibrocytes were confirmed by flow cytometry. Human fibrocytes were stained with control antibody (closed black) and specific antibodies (open gray) for collagen I, CXCR4 and CD45. B: Migration of human fibrocytes in response to CXCL12 and its inhibition by AMD3100. Fibrocytes ( $1 \times 10^4$ /well) were added in upper chamber with or without AMD3100 (1 µg/ml). Fibrocytes were tested for chemotaxis ( $n=3$ ) to CXCL12 (10 and 100 ng/ml). Data are shown as the mean  $\pm$  SD of three wells. Similar results were obtained in four separate experiments. \* $p < 0.001$  versus CXCL12 (100 ng/ml) alone.

### Treatment with AMD3100 reduces migration of fibrocytes into the lungs *in vivo*

Finally, we examined whether AMD3100 could inhibit the migration of fibrocytes into the lungs of bleomycin-treated mice. Administration of AMD3100 (6 mg/kg/day) significantly inhibited the number of CD45<sup>+</sup>Collagen I<sup>+</sup> fibrocytes in the lungs of mice treated with bleomycin on day 7 (bleomycin:  $28.6 \pm 10.6\%$  vs bleomycin+AMD3100:  $8.0 \pm 6.9$ ;  $p < 0.05$ ) (figure 6).



**Figure 6** Administration of AMD3100 reduces the number of fibrocytes into the lungs treated with bleomycin in mice. Mice were implanted with osmotic minipumps containing bleomycin (BLM). AMD3100 (6 mg/kg/day) was injected using osmotic minipump. Single-cell suspensions were isolated from the lungs of bleomycin-treated mice on day 7. After culturing in fibronectin-coated 10 cm-dish, the adherent cells were stained for CD45 and Collagen I, and examined by flow cytometry. A: Flow cytometric analysis of fibrocytes. The data shows the double staining with anti-CD45 and anti-collagen I antibodies for adherent cells of the lungs of bleomycin-treated mice. B: The percentage of CD45<sup>+</sup>Collagen I<sup>+</sup> fibrocytes in bleomycin-treated lungs. Data are shown as the mean  $\pm$  SD of 4 mice. \* $p < 0.05$ , significant differences between bleomycin alone and bleomycin+AMD3100 (6 mg/kg/day).

## DISCUSSION

In the present study, we demonstrated the antifibrotic effects of CXCR4 antagonist AMD3100 in bleomycin-induced pulmonary fibrosis in mice. AMD3100 showed the inhibitory effects of migration of fibrocytes in response to CXCL12 *in vitro* and *in vivo*. These results suggest the potential of AMD3100 to be an antifibrotic drug for treatment of patients with pulmonary fibrosis.

Circulating fibrocytes are thought to contribute organ fibrosis and wound healing (25). In the pulmonary fibrosis, Hashimoto *et al.* reported the contribution of bone marrow-derived cells in bleomycin-induced pulmonary fibrosis model (4). Phillips *et al.* also demonstrated the CD45<sup>+</sup>CXCR4<sup>+</sup>Collagen I<sup>+</sup> cells play a role in pulmonary fibrosis induced by bleomycin (5). Both reports indicated the critical role of CXCL12/CXCR4 axis for fibrocytes to



migrate into the lungs. Other chemokine/chemokine receptor systems are also known to play roles in trafficking of fibrocytes, whereas CXCL12/CXCR4 could be main axis in pulmonary fibrosis. In fact, CXCL12 was reported to be mostly expressed in macrophages and airway epithelial cells in the fibrotic lungs (26). Therefore controlling migration of fibrocytes by blockade of CXCR4 might lead to reduce pulmonary fibrosis. In fact, Phillips *et al.* showed that treatment with neutralizing antibody of CXCL12 ameliorated bleomycin-induced pulmonary fibrosis in mice (5). Thinking of clinical situation, a small molecule drug such as an inhibitor or antagonist of CXCR4 might be more useful for treatment of patients with pulmonary fibrosis. In the present study, we used a small molecule antagonist AMD3100, which was clearly demonstrated to be specific for CXCR4 and originally found to inhibit the replication of T-lymphotropic human immunodeficiency virus strains by blocking the entry into T cells via CXCR4 (27, 28). Administration of AMD3100 significantly reduced bleomycin-induced pulmonary fibrosis in mice. In addition, AMD3100 improved weight loss of mice treated with bleomycin in the late phase paralleled with the reduction of pulmonary fibrosis. Recently, Song *et al.* reported an inhibitory effect of CXC chemokine receptor 4 antagonist AMD3100 in bleomycin-induced pulmonary fibrosis model (29). They reported the elevation of numbers of lymphocytes and macrophages in bronchoalveolar lavage fluid (BALF). However, our results showed that AMD3100 did not affect the cells in BALF on day 7 and reduced the percentage of lymphocyte on day 14. The reason for this discrepancy remains unclear, but might be due to the difference in experimental systems used in both studies. In our study, bleomycin was administered systemically using osmotic minipump, but they used intratracheal route of injection of bleomycin. The systemic distribution of bleomycin more closely reflects the drug-induced lung toxicity in clinic. Since CXCR4 is expressed in some population of lymphocytes, it is not surprising that AMD3100 inhibited the migration of lymphocytes into the lungs.

Regarding the antifibrotic effects of AMD3100, our data was consistent to the report by Song *et al.* and extend their results by using another experimental method. In addition, AMD3100 significantly reduced the migration of fibrocytes into the lungs treated with bleomycin in mice. The direct effects of AMD3100 on migration of fibrocytes were confirmed by using human fibrocytes. The *in vivo*

experiment also demonstrated the reduced number of fibrocytes in the lungs treated with bleomycin and AMD3100. However, inhibition of the number of fibrocytes in the lungs by AMD3100 was partial, indicating that the other factors such as other chemokines may, at least in part, contribute to the migration of fibrocytes into the lungs *in vivo*.

Recently, the number of fibrocytes in peripheral blood of patients with pulmonary fibrosis is reported to correlate with the parameters of respiratory function test as well as prognosis of patients (30, 31). Further study would be required to understand the role of fibrocytes in pulmonary fibrosis in clinic.

These results suggest that AMD3100 could be an inhibitor of migration of fibrocytes and that treatment with AMD3100 might be useful approach for patients with pulmonary fibrosis.

## CONFLICT OF INTEREST

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