Dual Effect of AMD3100, a CXCR4 Antagonist, on Bleomycin-Induced Lung Inflammation

Masaki Watanabe, Wataru Matsuyama, Yuko Shirahama, Hideo Mitsuyama, Ken-ichi Oonakahara, Satoshi Noma, Ikkou Higashimoto, Mitsuhiro Osame and Kimiyoshi Arimura

*J Immunol* 2007; 178:5888-5898; doi: 10.4049/jimmunol.178.9.5888

http://www.jimmunol.org/content/178/9/5888

**References**
This article cites 62 articles, 27 of which you can access for free at:
http://www.jimmunol.org/content/178/9/5888.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

**Errata**
An erratum has been published regarding this article. Please see next page or:
/content/181/9/6670.full.pdf
Dual Effect of AMD3100, a CXCR4 Antagonist, on Bleomycin-Induced Lung Inflammation

Masaki Watanabe, Wataru Matsuyama, Yuko Shirahama, Hideo Mitsuyama, Ken-ichi Oonakahara, Satoshi Noma, Ikkou Higashimoto, Mitsuhiro Osame, and Kimiyoshi Arimura

The chemokine receptor CXCR4, which binds the chemokine stromal cell-derived factor 1, has been reported to be involved in the chemotaxis of inflammatory cells. In addition, AMD3100, an antagonist of CXCR4, has been reported to be an attractive drug candidate for therapeutic intervention in several disorders in which CXCR4 is critically involved. However, little is known about the therapeutic value of AMD3100 in the treatment of pulmonary fibrosis. In this study, we examined the effects of AMD3100 on a murine bleomycin-induced pulmonary fibrosis model. Concurrent administration of AMD3100 and bleomycin apparently attenuated bleomycin-induced pulmonary inflammation. In this process, an inhibition of neutrophil recruitment at early stage followed by the decrease of other inflammatory cell recruitment in the lung were observed. It also inhibited the expression of cytokines, including MCP-1, MIP-2, MIP-1α, and TGF-β. In contrast, when AMD3100 was administered following bleomycin treatment, the bleomycin-induced lung inflammation progressed and consequently resulted in severe pulmonary fibrosis. This process, an increase of inflammatory cell recruitment, an up-regulation of lung MCP-1 and TGF-β, and pathological importance of SDF-1/CXCR4 interactions (22). This makes AMD3100 an ideal tool to evaluate the physiological action of AMD3100 effect on pulmonary fibrosis using the murine model of bleomycin-induced pulmonary fibrosis. Interestingly, we found that AMD3100 has dual effect on bleomycin-induced pulmonary fibrosis. Difference of inflammatory cell recruitment and activation might be associated with the dual effect of AMD3100 on bleomycin-induced pulmonary fibrosis. Journal of Immunology, 2007, 178: 5888–5898.

Chemokines are small cytokine-like peptides that bind to their G protein-coupled cell-derived receptor. A number of leukocyte populations, including neutrophils, monocytes, lymphocytes, and eosinophils, express their G protein-coupled receptors (1, 2). Stromal cell-derived factor 1 (SDF-1/CXCL12) is a CXC chemokine that binds to the chemokine receptor CXCR4, which is highly expressed in several types of cancer cell and that their interaction contributes to cancer metastasis or dissemination into the hematopoietic, cardiovascular, and cerebellar systems (12, 13). Also, it has been demonstrated that CXCR4 and SDF-1 are highly expressed in several types of cancer cell and that their interaction contributes to cancer metastasis or dissemination into the hematopoietic, cardiovascular, and cerebellar systems (12, 13). In this study, we hypothesized that AMD3100 might be effective to control pulmonary inflammation and fibrosis, and examined AMD3100 effect on pulmonary fibrosis using the murine model of bleomycin-induced pulmonary fibrosis. Interestingly, we found that AMD3100 had dual effect on the bleomycin-induced lung injury.

Materials and Methods

This study, which was conducted using female C57BL/6 mice (weight, 17–20 g; age, 8 wk) in specific pathogen-free conditions and was approved by the Kagoshima University Ethics Committee for Animal Experiments.

Treatment of animals

The mice were anesthetized by i.p. administration of 1.5 mg of ketamine hydrochloride (Sankyo) and 0.3 mg of xylazine hydrochloride (Baycr), and the trachea was exposed via a cervical incision. Bleomycin (90 μg; Nippon Kayaku) was dissolved in 50 μl of saline and then instilled intratracheally with a 27-gauge needle. AMD3100 (Sigma-Aldrich) 200 μg in sterile 250 μl of PBS and U0126 (Promega) in 250 μl of 0.1% DMSO were injected i.p. for 10 days from day 0 or 1 after treatment with bleomycin on day 0.
We also administrated AMD3100 i.p. for 2 days from day 0 after treatment with bleomycin on day 0. Mice treated with PBS were used as controls. Anesthetized mice were sacrificed at the desired time point by axillary artery exsanguination.

Bronchoalveolar lavage fluid (BALF)

BALF was obtained by cannulating the trachea with a 20-gauge needle and infusing the lungs four times with 1 ml of saline. The recovery of BALF ranged between 2.0 and 3.5 ml, with no significant difference between the volume recovered from each mouse. The BALF cells were collected after centrifugation (1000 × g, 10 min, 4°C).

**Histological examination**

The excised lungs were immediately fixed with 10% formaldehyde neutral buffer solution for 48 h and then embedded in paraffin. Sagittal sections were cut at 4-μm thickness and stained with H&E and Masson-trichrome. The total lung area of the sections was used for fibrotic scale microscope evaluation (Olympus; BX50F4). The criteria for grading lung fibrosis were
in accordance with the method reported by Ashcroft et al. (25), as follows: grade 0, normal lung; grade 1, minimal fibrous thickening of alveolar or bronchiolar walls; grade 3, moderate thickening of walls without obvious damage to the lung architecture; grade 5, increased fibrosis with definite damage to the lung architecture and formation of fibrous bands or small fibrous masses; grade 7, severe distortion of architecture and large fibrous area; grade 8, total fibrous obliteration of the field. The severity of fibrotic changes in each lung section was assessed as the mean score for severity from the observed microscopic fields. The grade of lung fibrosis was scored on a scale from 0 to 8 by examining 10 randomly chosen regions per sample at a magnification of ×200 by four pathologists who were blinded to the treatment. After examination of the entire section, the mean of the scores from all the fields was considered as the fibrotic score (26).

Analysis of whole lung cells by flow cytometry

Whole lung tissues were pooled, mechanically macerated, constantly agitated in 0.2% (w/v) collagenase (type IV) in RPMI 1640 with 10% FBS for 60 min at 37°C, and finally washed in a balanced salt solution to remove aggregates. Lung cell suspensions were treated with ammonium chloride for RBC lysis. The cells were counted with a hemocytometer, and ~5 × 10⁶ cells were transferred to 5-ml polypropylene tubes (Sarstedt). After appropriate washing, cells were suspended in 50 μl of cold PBS containing 0.1% sodium azide, 10 mg/ml human IgG, and 20 μg/ml human IgG, and incubated for 10 min on ice, and with the appropriate dilutions of anti-mouse CXCR4 mAb (MAB21561; R&D Systems), anti-mouse Ly-6G mAb, or isotype-matched control IgG (R&D Systems) for an additional 15 min on ice. Cells were washed with PBS, and incubated with FITC-conjugated anti-rat IgG2 Ab (Acris Antibodies) for an additional 10 min on ice. Cells were washed with PBS, and then 7-aminoactinomycin D (BD Pharmingen) was added to each tube. The cells were washed with PBS, and subsequently analyzed by flow cytometry using a FACScan (BD Biosciences). Dead cells, determined by the incorporation of 7-aminoactinomycin D, were gated out. Results were processed using the CellQuest software (BD Biosciences).

Isolation of pulmonary neutrophils

The cell surface marker Ly-6G is highly expressed on mouse neutrophils. Therefore, we isolated Ly-6G-positive cells from the whole lung cells that we obtained, as described above. In each group, the Ly-6G-positive cells in
FIGURE 3. Histological change and BALF analysis. Although the histological changes on day 3 were similar between the two groups, i.e., mice treated with bleomycin alone and those administered AMD3100 from day 1, the bleomycin-induced pulmonary inflammation progressed and resulted in severe pulmonary fibrosis on day 21 in the mice treated with AMD3100 from day 1. In contrast, the infiltration of inflammatory cells was apparently decreased in the mice administered AMD3100 from day 0 or on days 0 and 1. On day 21, there were fewer inflammatory cells in the mice treated with AMD3100 from day 0 or on days 0 and 1 compared with the mice treated with bleomycin alone (representative data from 12 different mice in each group; original magnification, ×150; H&E staining; left panels in B, original magnification, ×300; H&E staining; right panels in B). After day 7, the total BALF cell count, and the macrophage, lymphocyte, and neutrophil counts were all significantly higher in the mice treated with AMD3100 from day 1 than in the mice treated with bleomycin alone (+, p < 0.01; **, p < 0.05, Bonferroni-Dunn test with one-way factorial ANOVA; n = 16 in each group; C). On day 3, both the total BALF cell count and the neutrophil count were significantly lower in the mice treated with AMD3100 from day 0 or on days 0 and 1 than in the mice treated with bleomycin alone (C). After day 7, the total BALF cell count and the macrophage and neutrophil counts were significantly lower in the mice treated with AMD3100 from day 0 or on days 0 and 1 than in the mice treated with bleomycin alone. After day 14, the total BALF cell count and the macrophage, lymphocyte, and neutrophil counts were significantly lower in the mice treated with AMD3100 from day 0 or on days 0 and 1 than in the mice treated with bleomycin alone (+, p < 0.01; **, p < 0.05, Bonferroni-Dunn test with one-way factorial ANOVA; n = 16 in each group; C).
whole lung were positively selected using magnetic beads (Miltenyi Biotec), according to the manufacturer’s protocol, and used for further analysis. Following selection, the Ly-6G-positive cells were also stained with May-Giemsa stain to identify the cell populations.

Western blot analysis

Lung tissues were homogenized on ice in 4 vol of lysis buffer composed of 50 mM NaCl, 20 mM Tris-HCl (pH 7.8), 50 mM NaF, 20 mM Na₃P₂O₇, 50 mM EGTA, 1 mM Na₃VO₄, 1% Triton X-100, and a mixture of protease inhibitors (Roche). Also, 1×10⁶ Ly-6G-positive pulmonary cells were lysed in 0.5 ml of lysis buffer, as described above. After centrifugation at 19,000 g for 20 min at 4°C, the supernatant was recovered. The supernatant was added to 10 µl of double-strength sample buffer (125 mM (pH 6.8), Tris-HCl (pH 7.4), 10% 2-ME, 4% SDS, 10% sucrose, and 0.004% bromphenol blue). The samples were boiled for 10 min. Eluted proteins were analyzed on 12% polyacrylamide gels by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes at 150 mA for 1.5 h using a semidry system. The membranes were incubated with rabbit polyclonal Abs against phosphorylated or nonphosphorylated p44/42 MAPK protein (Cell Signaling Technology), followed by secondary anti-Ig Ab coupled with HRP (Amersham Biosciences). Peroxidase activity was visualized by the Enhanced Chemiluminescence Detection System (Amersham).

Northern blotting

Total RNA was extracted from 1 mg of lung tissue by using TRIzol reagent, and Northern blotting was performed, as previously described (27). The cloning of mouse DDR1 cDNA and the sources of TGF-β, MCP-1, MIP-2, and MIP-1α codes were donated by T. Yoshimura (National Cancer Institute, Frederick, MD). Each cDNA was labeled with [α-³²P]dCTP using the Rediprime II random prime labeling system (Amersham Biosciences).

ELISA

The concentrations of TGF-β, MCP-1, MIP-2, MIP-1α, and SDF-1 in 1 mg of homogenized lung were measured by using ELISA kits or Duo kits (R&D Systems) in accordance with the manufacturer’s protocols.

FIGURE 4. Cytokine expression in lung. The mRNA expressions of MCP-1 and TGF-β in whole lung were significantly higher in the mice treated with AMD3100 from day 1 than in the mice treated with bleomycin alone. The mRNAs of MCP-1, MIP-2, MIP-1α, and TGF-β were significantly lower in the mice treated with AMD3100 from day 0 and 1 than in the mice treated with bleomycin alone (A: representative data from eight different mice in each group). The protein concentrations of MCP-1 and TGF-β in whole lung were significantly higher in the mice treated with AMD3100 from day 1 than in the mice treated with bleomycin alone. The protein concentrations of MCP-1, MIP-2, MIP-1α, and TGF-β were significantly lower in the mice treated with AMD3100 from day 0 or on days 0 and 1 than in the mice treated with bleomycin alone (*, p < 0.01, Bonferroni-Dunn test with one-way factorial ANOVA; n = 16 in each group; B).
Hydroxyproline assay

Hydroxyproline assay was performed, as described previously (26). Briefly, lung homogenate (0.5 ml) was digested in 1 ml of 6 N HCl for 8 h at 120°C. Five microliters of citrate/acetate buffer (5% citric acid, 7.24% sodium acetate, 3.4% sodium hydroxide, and 1.2% glacial acetic acid (pH 6.0)) along with 100 µl of chloramines T solution (282 mg of chloramines T, 2 ml of n-propanol, 2 ml of H2O, and 16 ml of citrate/acetate buffer (pH 6.0)) was added to µl of the sample and incubated for 20 min. Next, 100 µl of Ehrlich’s solution (2.5 g of (4-dimethylamino)benzaldehyde, 9.3 ml of n-propanol, and 3.9 ml of 70% perchloric acid) was added to each sample and incubated for 15 min at 65°C. The OD was determined at 550 nm on a DU 640 spectrophotometer (Beckman Instruments). Commercially available hydroxyproline (Sigma-Aldrich) was used to construct a standard curve.

Statistical analysis

The Mann-Whitney U test and Bonferroni-Dunn test with one-way factorial ANOVA were used. Kaplan-Meier analysis was used for survival analysis. A p value below 0.05 was considered to be significant. Values have been presented as the mean ± SD, unless otherwise stated.

Results

CXCR4-positive cells in bleomycin-treated mice and effect of AMD3100

As shown in Fig. 1, bleomycin-induced CXCR4-positive cell accumulation in lung peaked on day 3 and decreased (Fig. 1, A and B). To investigate the effect of AMD3100, we administrated different concentrations of AMD3100 everyday from day 0 to 2, and then counted CXCR4-positive cells on day 3. As shown in Fig. 1, AMD3100 inhibited bleomycin-induced CXCR4-positive cell accumulation in lung in a dose-dependent manner. We chose the concentration of 1 mg/ml of AMD3100 administration from day 1 significantly increased the pulmonary fibrosis score was significantly higher after day 7 than that of mice treated with bleomycin alone (p < 0.01, Fig. 2B), and the amount of hydroxyproline on day 21 was significantly higher than that of mice treated with bleomycin alone (p < 0.01, Fig. 2B). In contrast, when AMD3100 was administrated from day 0 or on days 0 and 1, the pulmonary fibrosis score was significantly lower after day 7 than that of mice treated with bleomycin alone (p < 0.01, Fig. 2B), and the amount of hydroxyproline on day 21 was significantly lower than that of mice treated with bleomycin alone (p < 0.01; **, p < 0.05, Bonferroni-Dunn test with one-way factorial ANOVA; n = 16 in each group; A). There was no significant difference in the SDF-1 concentration in whole lung among the four groups on days 3 and 7; however, on days 14 and 21, it was significantly higher in the AMD3100-administered groups than in the mice treated with bleomycin alone (*, p < 0.01; **, p < 0.05, Bonferroni-Dunn test with one-way factorial ANOVA; n = 16 in each group; B). AMD3100 administration on day 0 significantly decreased the Ly-6G-positive, CXCR4-positive cell recruitment in the lungs on day 1 (representative data from each group, C, *, p < 0.01, Bonferroni-Dunn test with one-way factorial ANOVA; n = 18 in each group; D).

FIGURE 5. CXCR4-positive cells and the amount of SDF-1 in whole lung. On days 3 and 7, the percentage of CXCR4-positive cells in whole lung was significantly lower in the AMD3100-administered groups than in the mice treated with bleomycin alone. On day 3, the percentage of CXCR4-positive cells in the mice treated with AMD3100 from day 0 was significantly higher than in the mice treated with AMD3100 from day 1. On day 7, the percentage of CXCR4-positive cells in the mice treated with AMD3100 from day 0 was significantly higher than in the mice treated with AMD3100 from day 1. On day 14, the percentage of CXCR4-positive cells in whole lung was significantly lower in the mice administered AMD3100 everyday than in the mice treated with bleomycin alone (**, * p < 0.01; ***, * p < 0.05, Bonferroni-Dunn test with one-way factorial ANOVA; n = 16 in each group; A). There was no significant difference in the SDF-1 concentration in whole lung among the four groups on days 3 and 7; however, on days 14 and 21, it was significantly higher in the AMD3100-administered groups than in the mice treated with bleomycin alone (**, * p < 0.01; ***, * p < 0.05, Bonferroni-Dunn test with one-way factorial ANOVA; n = 16 in each group; B). AMD3100 administration on day 0 significantly decreased the Ly-6G-positive, CXCR4-positive cell recruitment in the lungs on day 1 (representative data from each group, C, *, p < 0.01, Bonferroni-Dunn test with one-way factorial ANOVA; n = 18 in each group; D).
significantly lower than that of mice treated with bleomycin alone ($p < 0.01$, Fig. 2C).

**Histological analysis**

Histological examinations revealed that AMD3100 apparently modified the bleomycin-induced lung inflammation and fibrosis (Fig. 3A; day 21 in Fig. 3B). Although the histological changes on day 3 were almost similar between two groups of mice treated with bleomycin alone and AMD3100 administration from day 1, the bleomycin-induced pulmonary inflammation progressed and resulted in severe pulmonary fibrosis on day 21 in the mice treated with AMD3100 from day 1. In contrast, the infiltration of inflammatory cells was apparently decreased in the mice administrated AMD3100 from day 0 or on days 0 and 1. And on day 21, there were fewer inflammatory cells in the mice treated with AMD3100 from day 0 or on days 0 and 1 compared with the mice treated with bleomycin alone (Fig. 3B).

**BALF analysis**

After day 7, the total BALF cell count and the macrophage, lymphocyte, and neutrophil counts in whole lungs were all significantly higher in the mice treated with AMD3100 from day 1 than in the mice treated with bleomycin alone (Fig. 3C). On day 3, both the total BALF cell count and the neutrophil count were significantly lower in the mice treated with AMD3100 from day 0 or on days 0 and 1 than in the mice treated with bleomycin alone (Fig. 3C). After day 7, the total BALF cell count and the macrophage and neutrophil counts were significantly lower in the mice treated with AMD3100 from day 0 or on days 0 and 1 than in the mice treated with bleomycin alone. After day 14, the total BALF cell count and the macrophage, lymphocyte, and neutrophil counts were all significantly lower in the mice treated with AMD3100 from day 0 or on days 0 and 1 than in the mice treated with bleomycin alone.
Cytokine expression analysis

The mRNA and protein expressions of MCP-1 and TGF-β in whole lung were significantly higher in the mice treated with AMD3100 from day 1 than that in the mice treated with bleomycin alone (Fig. 4). The mRNA and protein expressions of MCP-1, MIP-2, MIP-1α, and TGF-β were significantly lower in the mice treated with AMD3100 from day 0 than that in the mice treated with bleomycin alone (Fig. 4).

CXCR4-positive cells and the amount of SDF-1 in lungs

We compared the number of CXCR4-positive cells in whole lung among the four groups. On days 3 and 7, the percentage of CXCR4-positive cells in whole lung was significantly lower in the AMD3100-administered groups than in the mice treated with bleomycin alone. On day 3, the percentage of CXCR4-positive cells in the mice treated with AMD3100 from day 1 was significantly higher than that in the mice treated with AMD3100 from day 0 or on days 0 and 1. On day 7, the percentage of CXCR4-positive cells in the mice treated with AMD3100 from day 1 was significantly higher than that in the mice treated with AMD3100 from day 0. On day 14, the percentage of CXCR4-positive cells in whole lung was significantly lower in the mice administered AMD3100 everyday than that in the mice treated with bleomycin alone (Fig. 5A). We next compared the amount of SDF-1 in whole lung among the four groups. There was no significant difference in the SDF-1 concentration in whole lung among the four groups on days 3 and 7; however, on days 14 and 21, the concentration was significantly higher in the AMD3100-administered groups than in the mice treated with bleomycin alone (Fig. 5B).

Because the administration of AMD3100 on day 0 was observed to be essential for its dual effect, and because LPS demonstrated that only the neutrophils in the AMD3100 group; therefore, we compared MAPK phosphorylation in pulmonary neutrophils in four groups. As shown in Fig. 6, the phosphorylation level of p38 MAPK and p44/42 MAPK was significantly decreased in the mice treated with AMD3100 from day 0 or on days 0 and 1 than in the mice treated with bleomycin alone (Fig. 6). The phosphorylation level of p44/42 MAPK was significantly higher in the mice treated with AMD3100 from day 1 than in the mice treated with AMD3100 alone (Fig. 6). There was no significant difference of p38 MAPK phosphorylation level between the mice treated with AMD3100 from day 1 and the mice treated with AMD3100 alone (Fig. 6). Also, there was no significant difference of JNK phosphorylation among four groups (Fig. 6).

Effect of p44/42 MAPK inhibitor, U0126

To examine the contribution of p44/42 MAPK activation in the amplification of bleomycin-induced lung inflammation by AMD3100 administration from day 1, we administrated p44/42 MAPK inhibitor, U0126, in combination with AMD3100 from day 1. As shown in Fig. 7, the survival rate on day 21 was significantly higher in the mice treated with U0126 than in the nontreated mice (*, p < 0.01, Kaplan-Meier analysis; n = 42 in each group; A). The amount of hydroxyproline on day 21 in the mice treated with U0126 was significantly lower than that in the nontreated mice (*, p < 0.01, Bonferroni-Dunn test with one-way factorial ANOVA; n = 12 in each group; B). U0126 apparently inhibited phosphorylation of p44/42 MAPK in the pulmonary neutrophils of the mice treated with AMD3100 from day 1 (representative data from 12 different mice in each group, C). U0126 significantly decreased the cytokine concentrations on day 14 in the whole lungs of the mice treated with AMD3100 from day 1 (*, p < 0.01, Bonferroni-Dunn test with one-way factorial ANOVA; n = 12 in each group; D).

Because the administration of AMD3100 on day 0 was observed to be essential for its dual effect, and because LPS demonstrated that only the neutrophils in the AMD3100 group; therefore, we compared MAPK phosphorylation in pulmonary neutrophils in four groups. As shown in Fig. 6, the phosphorylation level of p38 MAPK and p44/42 MAPK was significantly decreased in the mice treated with AMD3100 from day 0 or on days 0 and 1 than in the mice treated with bleomycin alone (Fig. 6). The phosphorylation level of p44/42 MAPK was significantly higher in the mice treated with AMD3100 from day 1 than in the mice treated with AMD3100 alone (Fig. 6). There was no significant difference of p38 MAPK phosphorylation level between the mice treated with AMD3100 from day 1 and the mice treated with AMD3100 alone (Fig. 6). Also, there was no significant difference of JNK phosphorylation among four groups (Fig. 6).

Effect of p44/42 MAPK inhibitor, U0126

To examine the contribution of p44/42 MAPK activation in the amplification of bleomycin-induced lung inflammation by AMD3100 administration from day 1, we administrated p44/42 MAPK inhibitor, U0126, in combination with AMD3100 from day 1. As shown in Fig. 7, the survival rate on day 21 was significantly higher in the mice treated with U0126 than in the nontreated mice (*, p < 0.01, Kaplan-Meier analysis; n = 42 in each group; A). The amount of hydroxyproline on day 21 in the mice treated with U0126 was significantly lower than that in the nontreated mice (*, p < 0.01, Bonferroni-Dunn test with one-way factorial ANOVA; n = 12 in each group; B). U0126 apparently inhibited phosphorylation of p44/42 MAPK in the pulmonary neutrophils of the mice treated with AMD3100 from day 1 (representative data from 12 different mice in each group, C). U0126 significantly decreased the cytokine concentrations on day 14 in the whole lungs of the mice treated with AMD3100 from day 1 (*, p < 0.01, Bonferroni-Dunn test with one-way factorial ANOVA; n = 12 in each group; D).

FIGURE 7. Effect of p44/42 MAPK inhibitor, U0126. Treatment with AMD3100 alone did not affect the survival of the mice. The survival rate on day 21 was significantly higher in the mice treated with U0126 than in the nontreated mice (*, p < 0.01, Kaplan-Meier analysis; n = 42 in each group; A). The amount of hydroxyproline on day 21 in the mice treated with U0126 was significantly lower than that in the nontreated mice (*, p < 0.01, Bonferroni-Dunn test with one-way factorial ANOVA; n = 12 in each group; B). U0126 apparently inhibited phosphorylation of p44/42 MAPK in the pulmonary neutrophils of the mice treated with AMD3100 from day 1 (representative data from 12 different mice in each group, C). U0126 significantly decreased the cytokine concentrations on day 14 in the whole lungs of the mice treated with AMD3100 from day 1 (*, p < 0.01, Bonferroni-Dunn test with one-way factorial ANOVA; n = 12 in each group; D).
Discussion

We demonstrated the dual effect of AMD3100, an antagonist of CXCR4, on bleomycin-induced lung inflammation. Inflammatory cells, such as eosinophils (6), lymphocytes (4), and neutrophils (15), express CXCR4, a receptor that contributes to the chemotaxis, activation, and homeostasis of these cells (4, 6, 16, 17). In addition, AMD3100 has been reported to be effective in preventing cancer metastasis (30), in treating collagen-unduced arthritis (22), and in attenuating allergic lung inflammation and airway hyperactivity in vivo (23). Therefore, we hypothesized that AMD3100 might attenuate or inhibit bleomycin-induced pulmonary inflammation. We found that the concurrent administration of AMD3100 and bleomycin apparently attenuated the pulmonary inflammation induced by bleomycin and significantly reduced the accumulation of neutrophils after day 3, macrophages after day 7, and lymphocytes after day 14 in lung. Moreover, it significantly reduced accumulation of CXCR4/Ly-6G-positive cells on day 1. Thus, concurrent AMD3100 administration reduces early phase accumulation of pulmonary neutrophils, followed by the decrease of other inflammatory cell accumulation in bleomycin-induced pulmonary inflammation. In contrast, when AMD3100 was administered following the bleomycin treatment, the bleomycin-induced inflammation progressed and resulted in severe pulmonary fibrosis, and p44/42 MAPK in the pulmonary neutrophils was activated. In addition, it significantly increased inflammatory cell accumulation, including neutrophil, macrophages, and lymphocytes after day 7. Increased neutrophil recruitment in lungs has been demonstrated to be a major cause of the increased mortality in bleomycin-induced pulmonary inflammation (31–34). Macrophages also play a pivotal role in the development of bleomycin-induced inflammation through MCP-1 (35) or TGF-β (36) and contribute to the development of bleomycin-induced pulmonary fibrosis (37). Taken together, the accumulation of inflammatory cell accumulation is likely to contribute to the dual effect of AMD3100 on bleomycin-induced pulmonary inflammation.

Concerning the role of TGF-β, we investigated the effects of AMD3100 on TGF-β expression in bleomycin-injected lungs. AMD3100 inhibited the intracellular calcium signaling and chemotactic response elicited by the natural CXCR4 ligand SDF-1 in murine bronchoepithelial cells, fibroblasts, and alveolar macrophages (47, 48). The inhibitory effect of concurrent AMD3100 administration might be associated with the decrease of the cytokines described above. In contrast, when AMD3100 was administered following the bleomycin treatment, the expression and production of MCP-1 and TGF-β were significantly up-regulated after day 7. MCP-1 is an important chemotactic factor of macrophages and plays an important role in the development of bleomycin-induced pulmonary fibrosis (35). The secretion of TGF-β from alveolar macrophages also plays a pivotal role in the development of bleomycin-induced pulmonary inflammation (36). We think that the alveolar macrophages also might contribute to the development of pulmonary fibrosis through these cytokine functions when AMD3100 was administered following the bleomycin treatment.

Concerning MAPK in pulmonary neutrophils, when AMD3100 was injected concurrently, the phosphorylation of p38 MAPK was significantly lower than in the mice treated with bleomycin alone. The p38 MAPK is activated in the lung tissue of bleomycin-induced pulmonary fibrosis in mice (28). In addition, the inhibition of p38 MAPK can ameliorate murine bleomycin-induced pulmonary fibrosis (49). Monocyte-derived macrophages secrete inflammatory chemokines, such as MCP-1 (22), in a p38 MAPK-dependent manner (50). Thus, p38 MAPK contributes to the phosphorilation of p38 MAPK in pulmonary neutrophils. Decreased phosphorylation of p38 MAPK in pulmonary neutrophils might contribute to the attenuation of bleomycin-induced pulmonary fibrosis when AMD3100 is administered following the bleomycin treatment. Moreover, in the mice treated with AMD3100 following bleomycin treatment, the expression and production of MCP-1 and TGF-β were significantly lower than in the mice treated with bleomycin alone. There was no significant difference of p38 MAPK phosphorylation level in pulmonary neutrophils between the mice treated with AMD3100 following bleomycin treatment and the mice treated with bleomycin alone. The p44/42 inhibitor, U0126, significantly abolished the enhanced pulmonary fibrosis by AMD3100 administration following bleomycin treatment and decreased the cytokine concentrations in whole lung. The p44/42 MAPK is rapidly activated when neutrophils are stimulated (52) and plays pivotal roles in LPS-induced acute lung injury (53). Activation of p44/42 MAPK is associated with neutrophil adhesion (54) and cytokine-induced delay of neutrophil apoptosis (55). The p44/42 MAPK signaling pathway is involved in the production of MIP-2 from rat neutrophil (56). Bleomycin up-regulates gene expression of angiotensin-converting enzyme, which is increased in fibrotic tissues and might play a pivotal role in fibrosis in cooperation with TGF-β (57–59), via p44/42 MAPK (60). Taken together, we think that p44/42 MAPK signaling pathway in pulmonary neutrophils might contribute to the dual effect of AMD3100 on bleomycin-induced lung inflammation.

The bicyclam AMD3100 was originally described as a highly potent and selective inhibitor of HIV-1 and HIV-2 replication (18). AMD3100 inhibited the intracellular calcium signaling and chemotactic response elicited by the natural CXCR4 ligand SDF-1 in different cell types (20, 61). AMD3100 also effectively inhibits autoimmune collagen-induced arthritis in mice, by specific interference with the SDF-1-mediated migration of Mac-1+/CXCR4+ leukocytes toward the inflamed joints (22). In addition, specific blocking of CXCR4 using AMD3100 reduced a number of pathological parameters related to asthmatic-type inflammation in lung
(23). Thus, AMD3100 is an attractive drug candidate for therapeutic intervention in several disorders in which CXCR4 is critically involved. CXCR4 is also reported to be involved in the recruitment of fibrocytes in pulmonary fibrosis (62). In this regard, our study showed an interesting effect of CXCR4 antagonist, AMD3100. Further studies addressing these points are necessary to evaluate the therapeutic value of CXCR4 antagonist, AMD3100, in pulmonary fibrosis.

Acknowledgment
We appreciate Rumi Matsumaya (Third Department of Internal Medicine, Kagoshima University Hospital, Kagoshima, Japan) for her excellent help.

Disclosures
The authors have no financial conflict of interest.

References


5898 AMD3100 MODULATES BLEOMYCIN-INDUCED LUNG INFLAMMATION

RETRACTED
01 NOVEMBER, 2008

by guest on June 9, 2017 http://www.jimmunol.org/ Downloaded from
Letter of Retraction

We wish to retract the article titled “Dual Effect of AMD3100, a CXCR4 Antagonist, on Bleomycin-Induced Lung Inflammation” by Masaki Watanabe, Wataru Matsuyama, Yuko Shirahama, Hideo Mitsuyama, Ken-ichi Oonakahara, Satoshi Noma, Ikkou Higashimoto, Mitsuhiro Osame, and Kimiyoshi Arimura, The Journal of Immunology, 2007, 178: 5888–5898.

This retraction follows an investigation by Kagoshima University into scientific misconduct by Dr. Wataru Matsuyama, the corresponding author of the article, which found that the article contains fabricated data. The investigation also found that Dr. Wataru Matsuyama was solely responsible for the scientific misconduct that resulted in the falsified or fabricated data in this paper.

We apologize to the scientific community for the need to retract the article.

Masaki Watanabe
Yuko Shirahama
Hideo Mitsuyama
Ken-ichi Oonakahara
Satoshi Noma
Ikkou Higashimoto
Mitsuhiro Osame
Kimiyo Arimura
Division of Respiratory Medicine
Respiratory and Stress Care Center
Kagoshima University Hospital
Kagoshima
Japan