Activation of Discoidin Domain Receptor 1 on CD14-Positive Bronchoalveolar Lavage Fluid Cells Induces Chemokine Production in Idiopathic Pulmonary Fibrosis

Wataru Matsuyama, Masaki Watanabe, Yuko Shirahama, Ken-ichi Oonakahara, Ikkou Higashimoto, Teizo Yoshimura, Mitsuhiro Osame and Kimiyoshi Arimura

http://www.jimmunol.org/content/174/10/6490

**References**
This article cites 55 articles, 23 of which you can access for free at:
http://www.jimmunol.org/content/174/10/6490.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/181/9/6672.full.pdf
Activation of Discoidin Domain Receptor 1 on CD14-Positive Bronchoalveolar Lavage Fluid Cells Induces Chemokine Production in Idiopathic Pulmonary Fibrosis

Wataru Matsuyama,* Masaki Watanabe,* Yuko Shirahama,* Ken-ichi Oonakahara,* Ikkou Higashimoto,* Teizo Yoshimura,† Mitsuhiro Osame,* and Kimiyoshi Arimura*

Discoidin domain receptor 1 (DDR1) is a receptor tyrosine kinase activated by collagen. We previously reported the functional expression of DDR1 on human monocyte-derived macrophages in vitro; however, information regarding its role in diseases is limited. Idiopathic pulmonary fibrosis (IPF) is a chronic lung disease, and the lesions contain an abundance of collagen. In this study, we examined DDR1 expression on bronchoalveolar lavage fluid (BALF) cells and investigated its functionality using samples obtained from 28 IPF patients, 13 chronic obstructive pulmonary disease patients, and 14 healthy volunteers. The DDR1 expression level in CD14-positive BALF cells was higher in IPF patients than in chronic obstructive pulmonary disease patients or healthy volunteers. The predominant isoform was DDR1b in the IPF group, while DDR1a and DDR1b were present in the other two groups. Using immunohistochemical analysis, we also detected DDR1 expression in inflammatory cells in the IPF lesion. In IPF patients, DDR1 activation induced the production of MCP-1, 4-1BB ligand, and matrix metalloproteinase-9 (MMP-9) from CD14-positive BALF cells in a p38 MAPK-dependent manner. In contrast, DDR1 activation of CD14-positive BALF cells in the other groups did not induce the production of these chemokines or MMP-9. These chemokines and MMP-9 contribute to the development of IPF and, therefore, we suggest that DDR1 might be associated with the pathogenesis of IPF in the tissue microenvironment. The Journal of Immunology.
early as 30 min after DDR1 activation, peaked at 60 min, and returned to basal levels by 120 min (16, 18, 19). In contrast, autophosphorylation of DDR1 was first detected 90 min after DDR1 activation, peaked at 4 h, and became undetectable by 12 h (19).

Furthermore, DDR1 is reported to be associated with matrix metalloproteinase-9 (MMP-9) production via a p38 MAPK-dependent pathway. DDR1b isoform being the predominant form, and that DDR1 activates p38 and c-Jun N-terminal kinase (JNK) (19). Therefore, we hypothesized that the alveolar macrophages of IPF patients might express functional DDR1. We investigated DDR1 expression and chemokine and MMP-9 production via a p38 MAPK-dependent pathway. DDR1 activation induced its autophosphorylation, followed by the recruitment of the DDR1 adaptor protein Shc. DDR1 activation also induced chemokine and MMP-9 production via a p38 MAPK-dependent pathway.

Materials and Methods

Flow cytometry analysis of BALF cells

This study was reviewed and approved by the Kagoshima University Faculty of Medicine Committee on Human Research. We investigated 28 IPF patients (mean age = 56.4 ± 12.3 years old, male:female = 14:14) and 13 chronic obstructive pulmonary disease (COPD) patients (mean age = 57.2 ± 14.3 years old, male:female = 8:6), and 14 healthy volunteers (mean age = 57.8 ± 17.8 years old, male:female = 8:6). All volunteers provided written informed consent.

The diagnosis of IPF was based on previously published international guidelines for COPD (23). A bronchofiberscope was wedged into the right B4 segment of the lung to collect BALF cells. Four 40-ml washes were performed with PBS containing 0.1% sodium azide, 10 ng/ml BSA, and 20 µg/ml human IgG and incubated for 10 min on ice. The cells were then incubated for an additional 15 min on ice with FITC-conjugated CD14 mAb (M5E2; BD Biosciences) and mouse anti-human CD29 mAb (MAR4, β1 integrin; BD Biosciences) or anti-human DDR1 mouse IgG mAb (48B3; Santa Cruz Biotechnology). The cells were washed with PBS and incubated with biotin-conjugated goat anti-mouse IgG Ab for 15 min on ice. Cells were then washed with PBS and incubated with PE-conjugated streptavidin for 15 min on ice. At the end of the incubation period, 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, identified by the 7-aminoactinomycin D incorporation, were gated out. Results were processed using the CellQuest software (BD Biosciences), as described previously (24).

BALF fluids were stored at −20°C for further analysis.

CD14-positive cells in BALF from each group were selected using magnetic beads (Miltenyi Biotec), according to the manufacturer’s protocol, and used for further analysis. Following selection, the CD14-positive cells were also stained with May-Giemsa stain to identify cell populations.

Immunohistochemistry

Biopsied lung tissues from 28 IPF patients were examined by immunohistochemistry. Cytokines were detected using a rabbit anti-DDR1 Ab (Santa Cruz Biotechnology). The tissue sections were incubated with 3% hydrogen peroxide solution in methanol for 10 min. Following a blocking reaction was performed, as reported previously (24). Sections were incubated with primary DDR1 Ab (R&D Systems) or anti-human CD14 (BD Biosciences) at a 1/50 dilution of the Ab. Negative control slides were incubated with rabbit IgG (R&D Systems). Secondary biotinylated anti-Ig Ab (R&D Systems) was added, and the mixture was incubated for 30 min at room temperature using a 1/50 dilution of the Ab. The immunohistochemical staining for DDR1 using a rabbit anti-DDR1 Ab (Santa Cruz Biotechnology) was detected using the diaminobenzidine (DAB) method, as described previously (25). Four-micrometer-thick sections were dehydrated, cleared, and mounted on poly(L-lysine)-coated slides, dewaxed, and washed with xylene and ethanol series. Endogenous peroxidase was quenched by incubation with 3% H2O2 in methanol for 10 min. Following incubation with primary DDR1 Ab, slides were rinsed with deionized water and incubated for 2 h at room temperature using a 1/50 dilution of the Ab. The secondary biotinylated anti-Ig Ab (R&D Systems) was added, and the mixture was incubated for 30 min at room temperature. The mixture was then rinsed with deionized water. The mixture was added, and the mixture was incubated for 10 min. A brown color reaction represented a positive result.

CLTSA

CD14-positive cells (1 × 10⁶ cells/ml) in BALF were incubated with 513DDR1 Ab (16, 18) or 50 ng/ml type I collagen (Sigma-Aldrich) and

Table 1: Clinical and clinical features in each group

<table>
<thead>
<tr>
<th>Feature</th>
<th>IPF Patients (n = 28)</th>
<th>COPD Patients (n = 13)</th>
<th>Healthy Volunteers (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male:female</td>
<td>20:8</td>
<td>8:6</td>
<td>6:8</td>
</tr>
<tr>
<td>Smoker/non-smoker</td>
<td>18:10</td>
<td>9:4</td>
<td>8:6</td>
</tr>
<tr>
<td>PaO₂ (mmHg)</td>
<td>65.1 ± 32.1</td>
<td>69.2 ± 29.8</td>
<td>89.1 ± 12.3</td>
</tr>
<tr>
<td>PaCO₂ (mmHg)</td>
<td>41.2 ± 12.3</td>
<td>49.2 ± 23.1</td>
<td>40.1 ± 10.1</td>
</tr>
<tr>
<td>VC (% of predicted value)</td>
<td>63.3 ± 13.8</td>
<td>88.3 ± 23.2</td>
<td>90.1 ± 15.7</td>
</tr>
<tr>
<td>FEV₁ (% of predicted value)</td>
<td>74.1 ± 11.3</td>
<td>65.1 ± 32.1</td>
<td>91.2 ± 12.3</td>
</tr>
<tr>
<td>FEV₁/VC (%)</td>
<td>0.95 ± 0.08</td>
<td>0.52 ± 0.12</td>
<td>0.96 ± 0.06</td>
</tr>
<tr>
<td>DLCO (%)</td>
<td>50.8 ± 16.9</td>
<td>80.3 ± 25.2</td>
<td>95.1 ± 10.2</td>
</tr>
<tr>
<td>Total cell count (x 10⁵/µl)</td>
<td>2.88 ± 1.99</td>
<td>2.91 ± 1.85</td>
<td>1.89 ± 1.67</td>
</tr>
<tr>
<td>Macrophage (x 10⁵/µl)</td>
<td>1.82 ± 1.66</td>
<td>1.76 ± 1.41</td>
<td>1.82 ± 1.62</td>
</tr>
<tr>
<td>Lymphocyte (x 10⁵/µl)</td>
<td>0.69 ± 0.44</td>
<td>0.61 ± 0.38</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>Neutrophil (x 10⁵/µl)</td>
<td>0.41 ± 0.15</td>
<td>0.66 ± 0.21</td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td>Total CD14⁺ (x 10⁵/µl)</td>
<td>69.3 ± 23.1</td>
<td>67.8 ± 22.1</td>
<td>65.3 ± 26.2</td>
</tr>
<tr>
<td>Total β₁ integrin (%)</td>
<td>34.1 ± 13.1</td>
<td>35.2 ± 14.1</td>
<td>35.1 ± 16.1</td>
</tr>
<tr>
<td>CD14 + β₁ integrin (%)</td>
<td>23.4 ± 12.3</td>
<td>25.2 ± 16.1</td>
<td>24.4 ± 15.1</td>
</tr>
<tr>
<td>MCP-1 (pg/ml)</td>
<td>67.1 ± 11.2</td>
<td>9.2 ± 10.1</td>
<td>9.8 ± 8.5</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>82.3 ± 23.1</td>
<td>28.2 ± 12.3</td>
<td>12.2 ± 10.1</td>
</tr>
<tr>
<td>MIP-1α (pg/ml)</td>
<td>59.2 ± 22.1</td>
<td>23.1 ± 18.9</td>
<td>12.1 ± 12.3</td>
</tr>
<tr>
<td>GM-CSF (pg/ml)</td>
<td>278.9 ± 87.2</td>
<td>98.2 ± 32.1</td>
<td>88.2 ± 34.1</td>
</tr>
</tbody>
</table>

*Includes exsmoker.

bp < 0.05.

*p < 0.05, by Bonferroni-Dunn test with one-way factorial ANOVA.
cultured for 24 h. Following culture, the supernatants were collected, and the concentrations of MCP-1, IL-8, MIP-1α, MMP-2, MMP-9, tissue inhibitor of metalloproteinases (TIMP)-1, and TIMP-2 were measured using ELISA kits (R&D Systems), according to the manufacturer’s protocols. To evaluate the effect of DDR1 integrin, another collagen receptor, we used DDR1 integrin neutralizing mAb (DE9, 10 μg/ml; Upstate Biotechnology), as previously described (16, 18).

To evaluate whether chemokine and cytokine productions induced by DDR1 activation are dependent on p38 MAPK, we pretreated CD14-positive BALF cells with 10 μM SB203580 (Biochem-Novabiochem) for 30 min, followed by stimulation with collagen or DDR1 agonistic Ab. We also measured MCP-1, IL-8, MIP-1α, and GM-CSF concentrations in the BALF from each group.

Cytokine concentrations were determined by linear regression from a standard curve using GraphPad software (Flow Laboratories), as described previously (27).

Gelatin zymography

MMP-2 and MMP-9 activities were analyzed using gelatin zymography. Precast 7.5% polyacrylamide minigels containing 0.3% SDS and gelatin (1 mg/ml) were obtained from the YAGAI Research Center. Fifteen microliters of undiluted culture supernatant was mixed with an equal volume of 50 mM Tris-HCl buffer (pH 6.8) containing 2% SDS, 10% glycerol, and 0.01% bromphenol blue. Samples were loaded and electrophoresed at 10 mA for 20 min, followed by electrophoresis at 20 mA for 80 min, until the dye front reached the bottom of the gel. As a positive control, partially activated MMP-9 and MMP-2 samples were also loaded on the gels. Following electrophoresis, the gels were agitated in 2.5% (v/v) Triton X-100 for 1 h to remove the SDS, followed by washing in 50 mM Tris-HCl buffer (pH 7.5) containing 200 mM NaCl for 1 h to restore the enzymatic activity. The gels were incubated for 24 h at 37°C in 50 mM Tris-HCl buffer (pH 7.5) containing 200 mM NaCl, 5 mM CaCl2, 0.02% (w/v) Brij-35, and 0.01% NaN3 to allow proteolysis of the gelatin substrate. Finally, the gels were stained for 1 h with 0.1% Coomassie brilliant blue G-25 in 30% methanol and 10% acetic acid, followed by destaining for 3 h in 30% methanol and 5% acetic acid. Gelatinolytic activity was identified as clear bands against the blue background.

Western blot analysis

To detect DDR1 isoforms, 1 × 10^7 CD14-positive BALF cells, which were selected using magnetic beads (Miltenyi Biotec), were lysed on ice for 20 min in 1 ml of lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1% Triton X-100, 10% glycerol, and a mixture of protease inhibitors (Roche). The lysates were centrifuged, and 20 μl of the supernatant was collected. Subsequently, 20 μl of double-strength sample buffer (20% glycerol, 6% SDS, and 10% 2-ME) was added to the supernatants. The samples were boiled for 10 min. Proteins were analyzed on 8% polyacrylamide gels by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes at 150 mA for 1 h using a semidyry system. The membranes were incubated with rabbit IgGs that specifically recognize DDR1a (16), DDR1b (18), both forms of DDR1 (Santa Cruz), or anti-human actin mouse IgG (Santa Cruz Biotechnology), followed by sheep anti-rabbit or mouse IgG coupled with HRP (Amersham). Peroxidase activity was visualized by...
the Enhanced Chemiluminescence Detection System (Amersham). The intensities of DDR1 isoforms and actin were analyzed using the NIH Image Program (National Institutes of Health), and then the relative amount of each DDR1 isoform (DDR1 amount ratio) in each patient was calculated.

DD1 autophosphorylation was analyzed, as previously described (16, 18, 19). First, we evaluated whether DDR1 in fresh CD14-positive BALF cells was activated by lysing $1 \times 10^7$ freshly isolated CD14-positive BALF cells, as described above. Subsequently, DDR1 in cell lysates was immunoprecipitated using anti-DDR1 Ab (C-20; Santa Cruz) and recombinant protein G-agarose (Invitrogen Life Technologies), as previously reported (16, 18, 19), and tyrosine phosphorylation of DDR1 was analyzed by Western blotting using a mouse monoclonal anti-phosphotyrosine IgG (4G10; Upstate Biotechnology) and a sheep anti-mouse IgG coupled with HRP (Amersham). Peroxidase activity was visualized by the Enhanced Chemiluminescence Detection System (Amersham).

In addition, to evaluate whether DDR1 activation by collagen or DDR1 agonistic Ab induces autophosphorylation of DDR1 and DDR1 signal transduction, $1 \times 10^7$ CD14-positive BALF cells were plated on dishes, serum starved in RPMI 1640 containing 1% FCS for 10 h, and subsequently activated with 50 μg/ml type I collagen (Sigma-Aldrich) or DDR1 agonistic Ab (513DDR1 Ab) (16, 18) and then cultured. Cell lysates were immunoprecipitated and analyzed, as described above.

To detect phosphorylation of p38 MAPK, CD14-positive cells were starved, as described above, and then activated with 50 μg/ml type I collagen (Sigma-Aldrich). Twenty microliters of cell lysate was directly mixed with 20 μl of sample buffer and analyzed. Phosphorylation of p38 MAPK was analyzed by Western blotting using rabbit polyclonal anti-phosphotyrosine (Upstate Biotechnology) and sheep anti-rabbit IgG HRP (Amersham). Peroxidase activity was visualized by the Enhanced Chemiluminescence Detection System (Amersham).

**Statistical analysis**

We used the Bonferroni-Dunn test with one-way factorial ANOVA (ANOVA). A p value below 0.05 was considered significant. Values were presented as the mean ± SD, unless stated otherwise.

**Results**

**BALF analysis in each group**

Table I shows the clinical features of the patients investigated in this study. There was no significant difference in terms of the male/female ratio, mean age, or percentage of smokers in the three groups. The COPD patients were significantly higher than in other groups (p < 0.05). Vital capacity (VC) and forced expiratory volume in 1 s (FEV1) were significantly lower in IPF patients than in other groups (p < 0.05, FEV1/VC, p < 0.01). BALF MCP-1, IL-8, MIP-1α, and GM-CSF levels in IPF patients were significantly higher than in COPD patients or healthy volunteers (p < 0.01). As shown in Fig. 1, the CD14-positive cells expressed DDR1. The percentage of DDR1-positive cells was significantly higher in IPF patients than in COPD patients or healthy volunteers. Almost all DDR1-positive cells were also CD14 positive. There was no significant difference among the three groups in the percentage of β1 integrin (another collagen receptor)-positive cells, CD14-positive cells, and β1 integrin-double-positive and CD14-positive cells in the total BALF cells (Table I).

**CD14-positive BALF cells from IPF patients expressed DDR1b**

In all three groups, the majority of CD14-positive cells were identified as macrophages by May-Giemsa staining (IPF patients: macrophages = 95.7 ± 5.4%; neutrophils = 4.6 ± 1.2%; COPD patients: macrophages = 95.8 ± 4.5%; neutrophils = 4.5 ± 1.3%; healthy volunteers: macrophages = 96.8 ± 4.1%; neutrophils = 3.9 ± 1.1%). As shown in Fig. 1, A and B, CD14-positive cells in all three groups express DDR1; however, there was a higher frequency of CD14-positive cells in the BALF from IPF patients (Fig. 1B). The total amount of DDR1 protein was also higher per $1 \times 10^7$ cells in IPF patients (Fig. 1C), and the proportion of cells expressing the DDR1b isoform was also much higher in the BALF from these patients (Fig. 1D). This experiment was repeated using samples from five different patients, and similar results were observed.

**DD1 autophosphorylation and recruitment of Shc in freshly isolated CD14-positive BALF cells of IPF patients**

To evaluate whether DDR1 activation occurs in vivo, we collected freshly isolated BALF CD14-positive cells and examined the phosphorylation levels of DDR1. As shown in Fig. 2, autophosphorylation and recruitment of Shc, the adaptor protein of DDR1b, were observed only in IPF patients and not in the other two groups. This experiment was repeated using samples from five different patients, and similar results were obtained.

**Immunohistochemical analysis of DDR1 expression**

As shown in Fig. 3, infiltrating inflammatory cells in the IPF lesion stained strongly with DDR1 Ab (Fig. 3A). Bronchial and alveolar epithelial cells were also positive for DDR1. Infiltrating inflammatory cells, including monocyte/macrophage-positive for CD14 (Fig. 3C), CD163-positive for MΦ (Fig. 3D), and acid-fast bacilli-positive for M. tuberculosis, showed similar results. DDR1 was co-expressed with CD14-positive cells from IPF patients and synergized pro-inflammatory chemokines from infiltrating inflammatory cells.

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Western blot analysis for DDR1 autophosphorylation in $1 \times 10^7$ fresh CD14-positive BALF cells. The membrane was probed with either anti-phosphotyrosine Ab, anti-DDR1 Ab (C-20), or anti-Shc Ab. Arrows indicate phosphorylated DDR1 or phosphorylated Shc. DDR1 autophosphorylation (upper arrow) and recruitment of Shc (lower arrow) were seen in IPF patients, but not in the other two groups. Representative data of five individual experiments using samples from five different donors are shown.
FIGURE 3. Immunohistochemistry of the biopsied lung of an IPF patient. Inflammatory cells in the IPF lesion showed strong positive staining for DDR1 (A). Bronchial and alveolar epithelial cells showed moderate positive staining for DDR1. Inflammatory cells in the IPF lesion also showed positive staining for CD14 as well (C) (A and B, ×300; C and D, ×500, original magnification; B, negative control for DDR1; D, negative control for CD14).

FIGURE 4. Effect of DDR1 activation on chemokine production in alveolar macrophages. A, DDR1 activation of alveolar macrophages from IPF patients induced MCP-1, IL-8, and MIP-1α production. DDR1 activation had no effect on COPD patients or healthy volunteers (*, p < 0.01, Bonferroni-Dunn test with one-way factorial ANOVA, in comparison with no activation in IPF patients; control IgM, control for DDR1 Ab). B, DDR1 activation showed a synergistic effect on LPS-induced chemokine productions from alveolar macrophages in IPF patients. In the presence of all LPS concentrations including 0 μg/ml, DDR1 activation significantly up-regulated the chemokine production (*, p < 0.01; **, p < 0.001, Bonferroni-Dunn test with one-way factorial ANOVA).
contrast, DDR1 stimulation did not induce nor up-regulate chemokine production from CD14-positive BALF cells from either the control or COPD patients. In preliminary experiments, we cultured CD14-positive cells for various time points (4, 8, 12, 24, 36, and 48 h) and concluded that culture for 24 h is the optimal because the chemokine levels peaked at 24 h and remained elevated in IPF patients (data not shown) and, in the other groups, collagen or DDR1 agonistic Ab stimulation did not up-regulate chemokine production. Furthermore, to evaluate the effect of microbeads, we cultured the isolated CD14-positive cells and cultured freshly obtained BALF cells of each group for various time points (4, 8, 12, 24, 36, and 48 h) without any stimulation. The chemokine concentrations between isolated CD14-positive cells and freshly isolated BALF cells in the culture supernatants were compared. There was no significant difference in the chemokine concentrations between CD14-positive cell supernatants and fresh BALF cell supernatants (data not shown). Taken together, we believe that the effect of microbeads was negligible and, therefore, we concluded that up-regulated chemokine production in IPF patients was induced by DDR1 activation and not by the effect of microbeads used for the CD14-positive selection. DDR1 activation alone also up-regulated LPS-induced chemokine production from CD14-positive cells from IPF patients (Fig. 4B). Our previous study showed that DDR1β, but not DDR1α, could induce or up-regulate chemokine production from human monocyte-derived macrophages (16). In this study, CD14-positive BALF cells from COPD patients and healthy volunteers did not express DDR1β. Thus, the results of this study support the previous reports (16, 19).

**FIGURE 5.** Effect of DDR1 activation on MMP production from alveolar macrophages. DDR1 activation of alveolar macrophages obtained from IPF patients induced MMP-9 production without any effect on MMP-2, TIMP-1, or TIMP-2 production (*, p < 0.01, Bonferroni-Dunn test with one-way factorial ANOVA, in comparison with no activation in IPF patients; b1 block Ab, β1 integrin-neutralizing Ab). B, Results of gelatin zymography using culture supernatants from alveolar macrophages obtained from IPF patients (black and white color). DDR1 activation of alveolar macrophages obtained from IPF patients induced active MMP-9 production.

**DDR1 stimulation induced active MMP-9 production in CD14-positive BALF cells from IPF patients**

As shown in Fig. 5A, collagen or DDR1 agonistic Ab stimulation also induced MMP-9 production from IPF patient’s CD14-positive BALF cells. In preliminary experiments, we cultured CD14-positive cells for various time points (4, 8, 12, 24, 36, and 48 h) and decided that culture for 24 h is the optimal time point because the production level of MMP-9 peaked at 24 h and decreased to basal level at 48 h, while the levels of MMP-2 and TIMPs did not change (data not shown). Active MMP-9 was produced from CD14-positive BALF cells (Fig. 5B). This active MMP-9 production was not inhibited by adding an Ab that blocks β1 integrin, another collagen receptor (10 μg/ml; Upstate Biotechnology). Neither collagen nor DDR1 agonistic Ab induced MMP-2, TIMP-1, or TIMP-2 production from CD14-positive BALF cells obtained from COPD patients or healthy volunteers (data not shown).

**Collagen and DDR1 agonistic Ab stimulation induced DDR1 autophosphorylation, recruitment of Shc, and p38 MAPK phosphorylation**

Based on our previous results (16, 18, 19), we predicted that DDR1β signal transduction might occur in the alveolar macrophages of IPF patients and chose 60 min as the stimulation time. As shown in Fig. 6A, collagen stimulation induced DDR1 autophosphorylation with recruitment of Shc in IPF patient cells, and this was not observed in the other two groups. As shown in Fig. 6B, collagen stimulation induced p38 MAPK phosphorylation in IPF patient cells, but not in the other two groups. The results of...
DDR1 agonistic Ab stimulation were the same as those obtained with collagen (data not shown). These experiments were repeated using samples from five different patients, and similar results were obtained.

p38 MAPK inhibitor (SB203580) significantly inhibited chemokine productions induced by DDR1 activation.

To evaluate whether the chemokine and MMP-9 production induced by DDR1 activation is dependent on p38 MAPK, we pretreated CD14-positive BALF cells with a p38 MAPK inhibitor (SB203580) for 30 min and then stimulated the cells with collagen or DDR1 agonistic Ab. As shown in Fig. 7, the p38 MAPK inhibitor significantly attenuated chemokine and MMP-9 production induced by DDR1 activation. Our data confirmed that SB203580 did not affect the viability of the cells (data not shown).

Discussion

The processes observed in IPF are associated with the production, deposition, and proteolysis of the ECM, which may lead to irreversible pulmonary structural remodeling with fibrosis (9). In IPF, the basement membranes, which are a specialized form of the ECM in lung, are thickened, resulting in a dense deposition of collagen (8, 28). The initiating cause is unknown, but alveolar epithelial injury appears to be a critical early event of IPF (29). During this event, alveolar macrophages are the major inflammatory cells recruited into alveolar spaces in patients with IPF (30). During this process, alveolar macrophages have to move into the lesion through ECMs that are composed of collagen, and they also have to encounter dense collagen deposition in the IPF lesion (31). In contrast, in a healthy state, it is difficult for alveolar macrophages to encounter ECMs because ECMs are continuous and closely attached to the basal surface of the bronchial or alveolar epithelial cells in normal lung (31, 32). In this study, CD14-positive BALF cells expressed endogenous DDR1, and the majority of BALF cells were macrophages. Therefore, we believe the majority of DDR1-positive, CD14-positive cells are alveolar macrophages. Taken together, we propose that DDR1 expressed on alveolar macrophages, but not expressed on bronchial and alveolar epithelial cells (32), can easily encounter its ligand, collagen, and transduce signaling events in IPF.

In this study, DDR1 activation of alveolar macrophages of IPF patients induced the secretion of chemokines, such as MCP-1, IL-8, and MIP-1α. These results are compatible with our previous results (16). MCP-1 is a major chemoattractant for monocytes in inflammation and immune responses (33). MCP-1 is detectable in the BALF of IPF patients (34) and has been suggested to be associated with the pathogenesis of IPF (35). Alveolar macrophages and epithelial cells are the main cellular source of MCP-1 production in IPF (36). IL-8 is a potent chemoattractant for neutrophils and plays a pivotal role in acute inflammation by recruiting and activating neutrophils (37). As a result, IL-8 is considered to play a prominent role in the attraction of neutrophils to the lung in IPF (38). In IPF, alveolar macrophages are considered to be the cellular sources of IL-8 (39). MIP-1α is known to regulate the trafficking and activation state of select subgroups of inflammatory cells, including lymphocytes (40). MIP-1α can modulate leukocyte adhesion to the endothelium and contributes to leukocyte recruitment into the lungs (41). In addition, it was reported that BALF MIP-1α levels were elevated in IPF (42). As described above, all chemokines investigated in this study play a pivotal role in the immunological pathogenesis of IPF. The present study also showed that DDR1 activation can dramatically synergize the LPS-induced chemokine production. Taken together, we propose that DDR1 might potentially contribute to the development of IPF in combination with other factors that induce chemokine production.
Our study also showed that DDR1 activation of CD14-positive BALF cells induced the secretion of MMP-9. MMPs are a family of zinc- and calcium-dependent endopeptidases capable of proteolytically degrading many of the components of the ECM (43), and TIMPs are the endogenous inhibitors of MMPs (44). In pulmonary diseases, MMPs are believed to be associated with wound repair of the human respiratory epithelium (45), while TIMPs are believed to be associated with irreversible pulmonary structure remodeling via myofibroblasts (46). The imbalance of these factors is thought to contribute to the development of interstitial lung diseases (47). In IPF, the alveolar macrophage is one of the cellular sources that produces MMP-9 (9) and contributes to lung remodeling in IPF (31). Furthermore, DDR1 was reported to be necessary for MMP-9 production (21). Taken together, we think that DDR1 might be associated with the lung remodeling in IPF via MMP-9 production from alveolar macrophages.

Alveolar macrophages expressed higher levels of DDR1 in IPF patients than in COPD patients or healthy volunteers. The predominant isoform in IPF was DDR1b, while DDR1a was predominant in the other two groups. In our previous reports, we found that GM-CSF could induce DDR1 expression on monocytes and increase the amount of DDR1b (16, 19). In the present study, the GM-CSF concentration in BALF was significantly higher in IPF patients than in other groups. Therefore, we think that the high GM-CSF concentration in BALF might be one of the reasons for the difference in total DDR1 levels and in the DDR1 isoform expression pattern between IPF patients and the other groups. Furthermore, alveolar macrophages of IPF patients are considerable different functionally and metabolically from alveolar macrophages obtained from normal subjects because previous studies reported the spontaneous release of chemotactic factors for recruiting inflammatory cells (48, 49). The difference in the DDR1 isoform expression pattern in alveolar macrophages might be associated with the functional difference between IPF and healthy individuals.

DDR1 activation might be a unique signaling molecule of p38 MAPK in IPF. In our study, DDR1 activation of CD14-positive BALF macrophages induced p38 MAPK activation both in vitro and also in a murine IPF model (15). In addition, phosphorylation of p38 MAPK can ameliorate bleomycin-induced pulmonary fibrosis (52). Thus, p38 MAPK activation in the pathogenesis of IPF is known; however, there is little information regarding the signaling pathway upstream of p38 MAPK. In our report, DDR1-induced chemokine and MMP-9 production in alveolar macrophages was dependent on the p38 MAPK pathway. Therefore, we suggest that DDR1 might be one of the upstream signaling molecules of p38MAPK in IPF.

The cell surface receptor, β1 integrin, is another well-known collagen receptor, and ligation of β1 integrin can induce the expression of proinflammatory cytokines in monocytes (53). However, the expression of β1 integrin is low or undetectable on monocytic-derived macrophages (54, 55), suggesting the presence of collagen receptors other than β1 integrins that may promote cytokine production in macrophages. Indeed, the present study showed a significant difference in the DDR1 expression level between IPF patients and healthy volunteers or COPD patients, while there was no significant difference between the percentage of β1 integrin-positive alveolar macrophages. Taken together, we think that DDR1, not β1 integrin, is the collagen receptor on alveolar macrophages that contributes to the pathogenesis of IPF.

In conclusion, we showed functional DDR1b expression on alveolar macrophages of IPF patients and proposed a possible association of DDR1 in the pathogenesis of IPF. To date, there have been many reports investigating the inflammatory mediators or molecular interaction between inflammatory mediators and inflammatory cells in IPF; however, there is little information regarding the molecular interaction between the microenvironment, such as ECM, and inflammatory cells. Therefore, further studies addressing this point are necessary to clarify the pathogenesis of IPF.

Acknowledgments
We appreciate Dr. Carole Galligan (University Health Network, Toronto, Ontario, Canada) for editing the manuscript. We also wish special thanks to Rumi Matsuyama for her excellent technical help for this study.

Disclosures
The authors have no financial conflict of interest.

References
Letter of Retraction


This retraction follows an investigation by Kagoshima University into scientific misconduct by Dr. Wataru Matsuyama, the first and 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
Ken-ichi Oonakahara
Ikkou Higashimoto
Mitsuhiro Osame
Kimiyo Arimura
Division of Respiratory Medicine
Respiratory and Stress Care Center
Kagoshima University Hospital
Kagoshima
Japan

Teizo Yoshimura
Laboratory of Molecular Immunoregulation
Center for Cancer Research
National Cancer Institute
Frederick, MD