Blockade of Interleukin-6 Receptor Alleviates Disease in Mouse Model of Scleroderma

Shun Kitaba,* Hiroyuki Murota,* Mika Terao,* Hiroaki Azukizawa,* Fumitaka Terabe,† Yoshihito Shima,‡ Minoru Fujimoto,† Toshio Tanaka,‡ Tetsuji Naka,† Tadamitsu Kishimoto,§ and Ichiro Katayama*

From the Department of Dermatology,* Course of Integrated Medicine and the Department of Respiratory Medicine, Allergy and Rheumatic Diseases,** Graduate School of Medicine, Osaka University, Osaka; the Laboratory for Immune Signal,† National Institute of Biomedical Innovation, Osaka; and the Laboratory of Immune Regulation,§ Osaka University Graduate School of Frontier Biosciences, Osaka, Japan

Activation of fibroblasts by interleukin-6 (IL-6) is implicated in the pathogenesis of scleroderma, suggesting that the inhibition of fibroblast activation may be a promising scleroderma treatment. In this study, we used an IL-6 blocking antibody (Ab) and IL-6 knockout (IL-6KO) mice to examine the role of IL-6 in the bleomycin (BLM)-induced mouse model of scleroderma. BLM was administered to C57BL/6 and IL-6KO mice to induce dermal sclerosis. BLM-treated and control phosphate-buffered saline-treated mice were treated with anti-mouse IL-6 receptor monoclonal Ab (MR16-1). Disease severity was evaluated by measuring dermal thickness and skin hardness, by counting the numbers of α-smooth muscle actin-positive cells and mast cells, and by examining the cutaneous draining lymph nodes. C57BL/6 mice with BLM induced scleroderma had elevated serum IL-6 levels and more severe dermal sclerosis than IL-6KO mice. Weekly administration of MR16-1, but not control Ab, prevented and improved dermal sclerosis, and also attenuated swelling of the draining lymph nodes. MR16-1 suppressed α-smooth muscle actin induction in IL-6–stimulated IL-6KO fibroblasts. Our results indicate that IL-6 contributes to BLM induced dermal sclerosis and that IL-6 receptor–specific monoclonal Ab may improve the symptoms of scleroderma by suppressing fibroblast activation. (Am J Pathol 2012, 180:165–176; DOI: 10.1016/j.ajpath.2011.09.013)
with intractable scleroderma treated with tocilizumab were previously reported to show marked amelioration of dermal sclerosis. Our results indicate that IL-6 induces dermal sclerosis via direct activation of dermal fibroblasts and that biomolecular targeting to suppress IL-6 might be a promising therapeutic approach for scleroderma.

**Materials and Methods**

**Dermal Fibroblast Isolation and Culture**

The dermis was collected and separated from the epidermis as described previously. Briefly, newborn wild-type and Il-6 knockout (KO) mouse pups (age 2 to 4 days) were sacrificed and rinsed in 70% ethanol. The skin was excised and treated with 4 mg/mL of dispase (Gibco, Invitrogen, Paisley, UK) for 1 hour at 37°C. The dermis was then separated from the epidermis, placed in phosphate-buffered saline (PBS) + 0.05% type-1 collagenase (Sigma-Aldrich, St. Louis, MO), and incubated at 37°C for 30 minutes with vigorous agitation to prepare single cells. After filtration, cells were resuspended in Dulbecco’s modified Eagle’s medium + 10% fetal bovine serum and incubated at 37°C and 5% CO₂.

The primary Il-6KO fibroblasts were passaged once or twice and used for subsequent experiments. Cells were confirmed to have the classical morphology (long spindle shape) of fibroblasts.

**Patients and Skin Samples**

This study included two scleroderma patients treated with tocilizumab and three scleroderma patients not treated with tocilizumab. Two scleroderma patients were treated with 8 mg/kg of tocilizumab monthly for 6 months with the permission of the Ethics Committee of Osaka University Hospital and after receipt of informed consent. Detailed patient information was described previously.

Skin samples were obtained from patients before and after treatment. Written informed consent was obtained from all patients before skin biopsy.

**Immunofluorescent Staining**

Wild-type (C57BL/6) and Il-6KO fibroblasts were cultured to semiconfluence in 350-mm culture plates. The cultures were fixed in 4% paraformaldehyde at room temperature for 10 minutes and permeabilized with 0.5% Triton in PBS for 5 minutes. The primary Abs used were mouse monoclonal anti-α-smooth muscle actin Ab (1:100, α-SMA; Dako-Cytomation, Carpinteria, CA), After 1 hour incubation, cells were stained for 30 minutes with Alexa Fluor 488 anti-mouse IgG secondary Ab to α-SMA (Invitrogen, Carlsbad, CA) and Hoechst 33342 (Molecular Probes, Eugene, OR). Mouse IgGₓ (Dako-Cytomation, Carpinteria, CA) was used as a control for nonspecific staining. Paraffin-embedded sections derived from scleroderma patients treated with 6 months of tocilizumab or 6 months of prednisolone, and redundant tissue from surgical specimens were deparaffinized and hydrated. Skin sections derived from patients were brought to a boil in 10 mmol/L sodium citrate buffer (pH 6.0) and then maintained at a subboiling temperature for 10 minutes. After blocking with 5% normal goat serum (Vector Laboratories, Burlingame, CA) in PBS, they were double-stained with mouse monoclonal anti-α-SMA Ab (Dako-Cytomation) and rabbit monoclonal phospho-p44/42 MAPK Ab (Cell Signaling, Beverly, MA). Secondary antibodies were as follows: anti-mouse Alexa Fluor 488 for α-SMA Ab and biotinylated anti-rabbit IgG (Vector Laboratories) plus DyLight594-conjugated Streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA) for phospho-p44/42 MAPK Ab. Images of immunolabeled sections were captured with a BZ-8000 microscope (Keyence, Osaka, Japan).

### Table 1. Effect of MR16-1 on BLM-Induced Dermal Sclerosis in a Prevention Model

<table>
<thead>
<tr>
<th>Thickness (mm)</th>
<th>1st (n = 4)</th>
<th>2nd (n = 4)</th>
<th>3rd (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Ab</td>
<td>0.137 ± 0.016</td>
<td>0.10 ± 0.011</td>
<td>0.123 ± 0.010</td>
</tr>
<tr>
<td>MR16-1</td>
<td>0.11 ± 0.01</td>
<td>0.111 ± 0.011</td>
<td>0.122 ± 0.013</td>
</tr>
<tr>
<td>% Change</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>81.82</td>
<td>106.33</td>
<td>98.65</td>
</tr>
<tr>
<td>BLM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Ab</td>
<td>0.33 ± 0.04*</td>
<td>0.36 ± 0.05*</td>
<td>0.30 ± 0.07*</td>
</tr>
<tr>
<td>MR16-1</td>
<td>0.22 ± 0.03†</td>
<td>0.29 ± 0.0225</td>
<td>0.16 ± 0.03†</td>
</tr>
<tr>
<td>% Change</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>67.09</td>
<td>81.29</td>
<td>52.22</td>
</tr>
</tbody>
</table>

*Values are mean ± SD. To quantify the impact of BLM treatment, % changes were calculated as follows: (evaluative consequences of BLM treatment)/that of PBS treatment) × 100 (%).

1 P < 0.01 PBS + Control Ab versus BLM + Control Ab.
2 P < 0.1 PBS + Control Ab versus BLM + MR16-1.
3 P < 0.05 PBS + MR16-1 versus BLM + MR16-1.

HPS, high-power field.
Western Blot Analysis

Wild-type and Il-6KO fibroblasts were prepared as described above and cultured to semiconfluence in 100-cm² culture plates. Before treatment, fibroblast cultures were washed twice with PBS, and culture media were replaced with low-serum (0.1% fetal bovine serum) Dulbecco’s modified Eagle’s medium containing 60 IU/mL penicillin, 100 IU/mL streptomycin, and 4 mmol/L glutamine. Low-serum medium was necessary to maintain viability of primary fibroblasts overnight.

Following 12 hours incubation in low-serum medium, treatments were applied to the cultures in fresh low-serum Dulbecco’s modified Eagle’s medium. Semiconfluent cultures were treated with 10 ng/mL of MR16-1 or 10 μg/mL of PD98058 (Calbiochem, San Diego, CA) for 3 hours, and then 10 ng/mL of recombinant mouse IL-6 (R&D Systems, Minneapolis, MN) was added to the cultures for 24 hours. At indicated time points, culture plates were washed twice with ice-cold PBS, and total cell protein was collected in 500 μL of lysis buffer [50 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1% deoxycholic acid, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1 mmol/L sodium orthovanadate, and protease inhibitor cocktail].

Western blot analysis was performed as previously described. Ten micrograms of protein were fractionated on SDS-polyacrylamide gels and transferred onto PVDF membranes (Bio-Rad, Hercules, CA). Nonspecific protein binding was blocked by incubating the membranes in 5% w/v nonfat milk powder in TBST [50 mmol/L Tris-HCl (pH 7.6), 0.1% v/v Tween-20]. The membranes were incubated with mouse monoclonal anti–α-SMA (Dako-Cytomation) Ab at a dilution of 1:10,000 for 60 minutes at room temperature. Protein bands were detected using the ECL Plus kit (GE Healthcare, Little Chalfont, UK). Western blot quantification was performed with ImageJ software (NIH, Bethesda, MD) and used to visualize fold expression differences between these treatment groups.

Mice and Induction of Skin Sclerosis

Six-week-old female mice were used in all experiments. C57BL/6 mice were purchased from Japan Clea (Osaka, Japan). Mutant C57BL/6 mice rendered null for IL-6 were described previously and were purchased from the National Institute of Biomedical Innovation (Osaka, Japan). Mice were maintained in our pathogen-free animal facility. All animal care was in accordance with the institutional guidelines of Osaka University. BLM (Nippon Kayaku, Tokyo, Japan) was dissolved in PBS at a concentration of 1 mg/mL and sterilized by filtration. BLM (0.1 mg/100 μL) was injected subcutaneously into the shaved back of the mice daily for 4 weeks with a 27-gauge needle as described by Yamamoto et al. Control mice received 100 μL of PBS instead.

RNA Isolation and Real-Time PCR

Sections of skin lesions and the cutaneous draining lymph nodes (LNs) were removed 1 day after the final injection. Total RNA was isolated using the SV Total RNA Isolation System (Promega, Madison, WI) and reverse transcribed into complementary DNA.

IL-6 expression was measured using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the mRNA. Sequence-specific primers were: IL-6, sense 5’-ACACACTGGTTCTGAGGGAC-3’; antisense 5’-TCCACAAGGTGCGAGGTTG-3’; GAPDH,

Table 1. Continued

<table>
<thead>
<tr>
<th>α-SMA–Positive Cells (Cells/HPS)</th>
<th>Mast Cells (Cells/HPS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st (n = 4)</td>
<td>2nd (n = 4)</td>
</tr>
<tr>
<td>6.00 ± 4.08</td>
<td>3.50 ± 1.29</td>
</tr>
<tr>
<td>5.00 ± 1.73</td>
<td>4.00 ± 1.41</td>
</tr>
<tr>
<td>83.33</td>
<td>114.29</td>
</tr>
<tr>
<td>104.0 ± 10.32</td>
<td>114.29</td>
</tr>
<tr>
<td>14.00 ± 1.83*</td>
<td>14.50 ± 4.93*</td>
</tr>
<tr>
<td>10.00 ± 1.41</td>
<td>7.00 ± 1.826</td>
</tr>
<tr>
<td>71.43</td>
<td>48.28</td>
</tr>
<tr>
<td>58.22 ± 6.88</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Effect of MR16-1 on BLM-Induced Dermal Sclerosis in a Treatment Model

<table>
<thead>
<tr>
<th></th>
<th>Thickness (mm)</th>
<th>Hardness (arbitrary)</th>
<th>α-SMA-positive cells (cells/HPS)</th>
<th>Mast cells (cells/HPS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st (n = 4)</td>
<td>2nd (n = 3)</td>
<td>1st (n = 4)</td>
<td>2nd (n = 3)</td>
</tr>
<tr>
<td>PBS Control Ab</td>
<td>0.14 ± 0.02</td>
<td>0.12 ± 0.03</td>
<td>4.80 ± 0.47</td>
<td>6.19 ± 1.29</td>
</tr>
<tr>
<td>MR16-1</td>
<td>0.12 ± 0.01</td>
<td>0.107 ± 0.006</td>
<td>4.67 ± 0.47</td>
<td>5.68 ± 0.43</td>
</tr>
<tr>
<td>% Changes</td>
<td>83.64</td>
<td>86.49</td>
<td>97.22</td>
<td>91.78</td>
</tr>
<tr>
<td>BLM Control Ab</td>
<td>0.30 ± 0.03*</td>
<td>0.29 ± 0.03*</td>
<td>9.34 ± 1.58*</td>
<td>9.81 ± 1.17†</td>
</tr>
<tr>
<td>MR16-1</td>
<td>0.21 ± 0.05†</td>
<td>0.18 ± 0.03§</td>
<td>5.46 ± 0.62†</td>
<td>5.41 ± 0.77§</td>
</tr>
<tr>
<td>% Changes</td>
<td>70.00</td>
<td>62.50</td>
<td>58.46</td>
<td>55.12</td>
</tr>
</tbody>
</table>

Mean ± SD was presented. To quantify the impact of BLM treatment, % changes were calculated as follows: (evaluative consequences of BLM treatment)/that of PBS treatment) × 100 (%).

Table 3. Attenuated BLM-Induced Dermal Sclerosis in Il-6KO Mice

<table>
<thead>
<tr>
<th></th>
<th>Thickness (mm)</th>
<th>Hardness (arbitrary)</th>
<th>α-SMA-positive cells (cells/HPS)</th>
<th>Mast cells (cells/HPS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st (n = 4)</td>
<td>2nd (n = 3)</td>
<td>1st (n = 4)</td>
<td>2nd (n = 3)</td>
</tr>
<tr>
<td>WT PBS</td>
<td>0.112 ± 0.013</td>
<td>0.14 ± 0.04</td>
<td>4.91 ± 0.38</td>
<td>5.46 ± 0.93</td>
</tr>
<tr>
<td>BLM</td>
<td>0.32 ± 0.02*</td>
<td>0.29 ± 0.06†</td>
<td>12.38 ± 0.81*</td>
<td>9.69 ± 0.51*</td>
</tr>
<tr>
<td>% Change</td>
<td>282.85</td>
<td>203.13</td>
<td>251.99</td>
<td>177.28</td>
</tr>
<tr>
<td>WT BLM</td>
<td>0.111 ± 0.010</td>
<td>0.12 ± 0.01</td>
<td>5.37 ± 0.48</td>
<td>5.12 ± 0.71</td>
</tr>
<tr>
<td>BLM</td>
<td>0.22 ± 0.03‡</td>
<td>0.17 ± 0.03§</td>
<td>7.14 ± 0.96†</td>
<td>5.86 ± 0.21‡</td>
</tr>
<tr>
<td>% Change</td>
<td>195.71</td>
<td>133.81</td>
<td>132.87</td>
<td>114.21</td>
</tr>
</tbody>
</table>

Mean ± SD was presented. To quantify the impact of BLM treatment, % changes were calculated as follows: (evaluative consequences of BLM treatment)/that of PBS treatment) × 100 (%).

Enzyme-Linked Immunosorbent Assay of IL-6 Levels in Sera and Conditioned Media

Serum samples were obtained from mice injected with BLM or PBS for 28 days. Conditioned media were obtained from cultured primary dermal fibroblasts of wild-type and Il-6KO mice after 24 hours. Serum and conditioned media IL-6 level was measured by enzyme-linked immunosorbent assay using a commercial kit (R&D Systems, Minneapolis, MN) with a detection limit of 7.8 pg/mL.

Vesmeter Measurements

Skin hardness was measured using a Vesmeter. Mice were sacrificed 1 day after the final injection. Skin hardness was measured three times at the injection area, avoiding the backbone of the mouse. Skin hardness was expressed as the area of the depression caused by the
with 1 mg/mL BLM (100 der paraffinized sections stained with 1% Toluidine Blue, and treatment/that of PBS treatment) was determined at the epidermal–dermal junction to dermal–fat junction) was determined using a microtome. Sections were stained with hematoxylin.

**Histopathological Analysis**

The back skin was removed 1 day after the final injection. Skin pieces were fixed with 10% formaldehyde for 24 hours, embedded in paraffin, and sectioned at 3-μm thickness using a microtome. Sections were cut and processed as described above. Sections were stained with hematoxylin.

**Immunohistochemical Analysis of α-SMA**

Sections were cut and processed as described above. For immunohistochemical analysis, sections were deparaffinized by passage through xylene and graded ethanol.

nols. Next, endogenous peroxide was blocked using 3% H2O2 in methanol for 5 minutes. Slides were blocked with 2% bovine serum albumin for 10 minutes, and stained with primary Ab (anti–α-SMA Ab 1:100 dilution) for 60 minutes. After washing with PBS containing 0.05% Triton, they were incubated with horseradish peroxidase (Dako-Cytomation) for 30 minutes, and counterstained with hematoxylin. α-SMA–positive fibroblastic cells were counted in 10 randomly selected sites under ×400 power using light microscopy.

**Flow Cytometric Analysis**

The skin draining LNs were assessed as a mixture to facilitate analysis. One day after the final infection, mice were sacrificed, and axillary, brachial, and inguinal LNs from each mouse were combined. Cell suspensions of LN cells were stained with antibodies against the following cell surface antigens: CD4, CD8, B220, CD11c, F4/80, and PDCA1 (BD Biosciences, San Jose, CA). Stained cells were analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences).

---

### Table 4. Number of Lymph Node Cells in the Scleroderma Mouse Model

<table>
<thead>
<tr>
<th></th>
<th>1st experiment (total number/lymph node, × 10⁶)</th>
<th>2nd experiment (total number/lymph node, × 10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS BLM % Change</td>
<td>PBS BLM % Change</td>
</tr>
<tr>
<td><strong>WT</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control Ab</td>
<td>0.97 ± 0.21 (n = 3)</td>
<td>2.17 ± 0.31† (n = 3)</td>
</tr>
<tr>
<td>MR16-1</td>
<td>0.76 ± 0.02 (n = 3)</td>
<td>1.85 ± 0.18 (n = 4)</td>
</tr>
<tr>
<td><strong>Il-6KO</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>0.81 ± 0.18 (n = 3)</td>
<td>1.53 ± 0.40‡ (n = 3)</td>
</tr>
<tr>
<td>Il-6KO</td>
<td>0.66 ± 0.26 (n = 3)</td>
<td>1.53 ± 0.20§ (n = 3)</td>
</tr>
</tbody>
</table>

Mean ± SD was presented. To quantify the impact of BLM treatment, % changes were calculated as follows: (evaluative consequences of BLM treatment) / (PBS treatment) × 100.

**Figure 1.** IL-6 production in BLM-treated C57BL/6 mice. C57BL/6 mice treated with PBS or BLM for 4 weeks. A: Serum samples were obtained from mice injected with 1 mg/mL BLM (100 μL/day, n = 11) or PBS (100 μL/day, n = 11) for 4 weeks. The data presented are from three experiments of three to five mice each for a total of 11 BLM-treated mice and 11 PBS-treated mice. Serum IL-6 level was measured by enzyme-linked immunosorbent assay using a kit with a detection limit of 7.8 pg/mL (R&D Systems). The mice from different experiments were given different symbols.

A: Serum samples were obtained from mice injected with 1 mg/mL BLM (100 μL/day, n = 11) or PBS (100 μL/day, n = 11) for 4 weeks. The data presented are from three experiments of three to five mice each for a total of 11 BLM-treated mice and 11 PBS-treated mice. Serum IL-6 level was measured by enzyme-linked immunosorbent assay using a kit with a detection limit of 7.8 pg/mL (R&D Systems). The mice from different experiments were given different symbols. B and C: Expression of IL-6 mRNA was measured by real-time PCR.
Computation Methods and Statistical Analysis

All data except change ratios are expressed as mean values ± standard deviations (SDs). To quantify the impact of MR16-1 and BLM treatment, change ratios (%) are calculated for single experiments in Table 1–4. Percent changes in Table 1 are averaged for three experiments and expressed as mean values ± standard errors (SEs). Unpaired t-test was used to examine the statistical value between two variable quantities. One-way analysis of variance and the Bonferroni post hoc multiple comparison procedure were used to determine the level of significance between each of three or more variable quantities.

Results

Elevated IL-6 in Mice with BLM-Induced Scleroderma

We first determined the serum concentration and mRNA expression of IL-6 in the skin and cutaneous draining LNs from mice with skin fibrosis induced by subcutaneous administration of BLM. The follow-up results are shown in Figure 2.
BLM injection. Serum IL-6 levels were undetectable by enzyme-linked immunosorbent assay in all PBS-treated mice, and in 3 of 11 C57BL/6 mice treated with BLM. However, IL-6 was detectable, thus elevated, in 8 of 11 BLM-treated mice (Figure 1A), with a mean of $11.9 \pm 5.24$ pg/mL ($n = 8$). IL-6 mRNA expression showed a trend toward increased levels in the skin of mice treated with BLM that was not statistically significant (Figure 1B), and was significantly elevated ($P < 0.05$) in the cutaneous draining LNs of BLM-treated mice relative to PBS-treated mice (Figure 1C). These results are consistent with a role for IL-6 in the pathogenesis of scleroderma in the BLM-induced mouse model.

**MR16-1 Prevents BLM-Induced Dermal Sclerosis**

We next investigated whether MR16-1, a rat anti-mouse IL-6 receptor monoclonal Ab, could ameliorate the dermal thickening and skin hardening symptoms observed in BLM-treated mice. Figure 2A shows the administration schedule of preventive intervention. Dermal thickness was significantly increased at the BLM injection site of control Ab–treated mice, but nearly normal at the PBS injection site of control Ab–, or MR16-1–treated mice. Importantly, BLM-induced dermal thickening was significantly attenuated by prophylactic administration of MR16-1 (Figure 2, B and C, Table 1).

Skin hardness in the BLM-injected group that was given MR16-1 was also significantly reduced compared to the BLM-injected group that was given the control Ab. The ameliorating effect of MR16-1 on skin hardness was relatively strong compared with the effect on dermal thickness (Figure 2, B and D, Table 1).

To further examine the effects of MR16-1 treatment, the numbers of $\alpha$-SMA–positive fibroblasts (termed myofibroblasts) (Figure 2, E and F, Table 1) and mast cells (Figure 2, G and H, Table 1), both key players in sclerosis of skin lesions, were evaluated. The numbers of myofibroblasts and mast cells were significantly increased in BLM-injected mice treated with control Ab relative to PBS-injected mice treated with control Ab. In BLM-injected mice treated with MR16-1, the numbers of myofibroblasts and mast cells were decreased significantly compared to the control value (BLM-injected mice treated with control Ab) (Figure 2, E to H, Table 1). These results suggest that treatment with MR16-1 might be effective during the fibrosing phase of scleroderma.

**MR16-1 Improves BLM-Induced Dermal Sclerosis**

Figure 3A shows the administration schedule of treatment intervention. As expected, the dermal thickness and skin hardness induced by BLM were diminished by therapeutic administration of MR16-1 compared with control Ab (Figure 3, B to D). The numbers of myofibroblasts and mast cells in lesional skin were also decreased by administration of MR16-1 compared with control Ab (Figure 3, E and F, Table 2). These results indicate that IL-6 may contribute to the pathogenesis of BLM-induced scleroderma and that blockade of IL-6 receptor may be a novel treatment of scleroderma.

**IL-6 Directly Modulates $\alpha$-SMA Expression in Dermal Fibroblasts in Vitro**

We next focused on whether dermal fibroblasts are a target of IL-6. Nontreated primary dermal fibroblasts from wild-type mice already express $\alpha$-SMA, and stimulation

---

**Figure 3.** Effect of MR16-1 on BLM-induced dermal sclerosis in a treatment model. A: Experimental protocol for treatment of BLM-induced dermal sclerosis by administration of MR16-1 or control Ab to either PBS- or BLM-treated mice ($n = 3$–4 for each group). The effect of Ab therapy was assessed on day 56. B: H&E staining of specimens derived from PBS- or BLM-injected mice treated with MR16-1 or control Ab (original magnification, $\times 40$), and measurements of dermal thickness (C) (the measurement region of dermal thickness was indicated with the length of each two-headed arrow in B) and skin hardness (D). The number of $\alpha$-SMA-positive fibroblasts (E) and mast cells (F) per HPF ($\times 400$) were determined by observation of 10 random grids. The value graphed is the average of the observation of 10 grids for each of the four mice in the group. C to F: Bars represent mean $\pm$ SD. *$P < 0.05$, **$P < 0.01$, one-way analysis of variance and Bonferroni post hoc multiple comparison. Data presented are from the first of two independent experiments with similar results. See Table 2 for data from both experiments.
with exogenous recombinant mouse IL-6 (rmIL-6) did not alter α-SMA expression (Figure 4A). Further, highly expressed levels of endogenous IL-6 from nontreated cultured primary wild-type dermal fibroblasts and decreased levels of α-SMA mRNA expression after MR16 treatment indicated that hyporesponsiveness of cultured primary wild-type dermal fibroblasts to exogenous IL-6 was presumably due to the autocrine regulation of α-SMA by IL-6 (Figure 4B). Thus, we switched to primary Il-6 KO mouse–derived fibroblasts and evaluated α-SMA expression using immunofluorescent staining and Western blot analysis. Low-level expression was observed in non-treated Il-6 KO dermal fibroblasts, but stimulation with 1 or 10 ng/mL of rmIL-6 induced α-SMA expression in a dose-dependent manner (Figure 4A). α-SMA expression was used to determine fold changes in expression by densitometry. Cultured dermal fibroblasts from wild-type (WT) and Il-6 KO mice were treated with 0, 1, and 10 ng/mL of rmIL-6 for 24 hours. These experiments were repeated three times, and the results of densitometric analyses are presented as the fold change (mean ± SD) compared with control. **P < 0.01, one-way analysis of variance and Bonferroni post hoc multiple comparisons. B: IL-6 levels in supernatants of cultured dermal fibroblasts from WT and Il-6 KO mice (left) after 24 hours. MR16-1 treatment decreased α-SMA mRNA expression in cultured primary WT dermal fibroblasts (right). C: MR16-1 and ERK inhibitor, PD98058, attenuated rmIL-6–induced α-SMA protein expression in cultured Il-6 KO fibroblasts. β-Actin expression was used to determine fold changes in expression by densitometry. These experiments were performed three times, and the results of densitometric analyses are presented as the fold change (mean ± SD) compared with control. **P < 0.01, *P < 0.05, one-way analysis of variance and Bonferroni post hoc multiple comparisons. D: Immunofluorescent staining for phosphorylated ERK (p-ERK, red) and α-SMA (green) in lesional skin derived from two tocilizumab-treated patients with scleroderma. A representative image of p-ERK, α-SMA fibroblasts (original magnification, ×1200) is shown. The number of p-ERK–positive α-SMA–positive fibroblasts per HPF (×400) was determined by observation of 10 random grids. Scale bar = 50 μm. The ratio of p-ERK–positive fibroblasts was calculated as follows: the number of p-ERK–, α-SMA+ fibroblasts/the number of p-ERK–, α-SMA– fibroblasts.
itive dermal fibroblasts in lesional skin of scleroderma patients. The number of Erk-activated α-SMA-positive cells in lesional skin in scleroderma patients treated with tocilizumab for 6 months was reduced to a similar level as in healthy skin, whereas the number in scleroderma patients treated with 10 mg/day of prednisolone for 6 months without tocilizumab was diminished in one patient and increased in two patients (Figure 4D).

**Attenuated BLM-Induces Dermal Sclerosis in Il-6KO Mice**

To investigate the role of IL-6 in BLM-induced dermal sclerosis, Il-6KO mice received subcutaneous injection of BLM or PBS for 4 weeks, and histological and physical examination of the lesional skin was performed (Figure 5A, Table 3). We found that BLM-induced dermal sclerosis in Il-6KO mice was attenuated compared with that in wild-type mice. Lack of visible changes in the skin between PBS-treated Il-6KO mice and PBS-treated wild-type mice indicated that IL-6 might not be involved in dermal homeostasis (Figure 5A). After 4 weeks of BLM treatment, dermal thickness and skin hardness in Il-6KO mice were significantly attenuated compared to wild-type mice (Figure 5A). The numbers of α-SMA-positive cells and mast cells in BLM-treated Il-6KO mice were significantly reduced compared to BLM-treated wild-type mice (Figure 5B). Table 3 summarizes the data from two experiments. These results indicate that IL-6 is likely to play an important role in promoting the fibrogenic responses elicited by BLM treatment.

**Enlarged Draining LNs Are Reduced in Size by a Block of IL-6 in the Mouse Model and in a Patient with Scleroderma**

We found that cutaneous draining LNs were visibly enlarged by BLM treatment in the scleroderma model mice, but not by PBS treatment (Figure 6A, Table 4). The total LN cell count per LN in control Ab–treated BLM-injected mice was significantly increased compared with control Ab–treated PBS-injected mice, and decreased by administration of MR16-1 to BLM-injected mice. Although it was only from a single experiment with a small number of mice, the weight per LN also showed similar findings to the results of the total LN cell count per LN. However, no histological differences were observed between LNs from BLM- and PBS-injected control Ab–treated mice (Figure 6A). Detailed cell fractionation analysis (using cell-surface antigens CD4, CD8, B220, CD11c, F4/80, and PDCA1) of cells isolated from the draining LNs revealed that the ratio of PDCA1+CD11c+ double-positive cells [plasmacytoid dendritic cells (pDCs)] was significantly increased in the draining LNs of prophylactically MR16-1–treated model mice (Figure 6B). Further, draining LNs were not grossly enlarged in BLM-treated Il-6KO mice (Figure 6C, Table 4), consistent with weight and total cell count per LN measurements in the normal range (Figure 6C).

**Discussion**

Our study demonstrates the critical role of IL-6 in dermal sclerosis. Blockade of IL-6 receptor with MR16-1 in the BLM-treated mice alleviates dermal sclerosis. This report also addresses outstanding problems in scleroderma pathogenesis, including the target of IL-6.

The source(s) of the elevated IL-6 in the sera of patients with scleroderma are still unclear. Several lines of
Evidence suggest peripheral blood mononuclear cells are a source. The supernatant concentration of IL-6 was reported to be statistically significantly elevated in peripheral blood mononuclear cells\(^6,20,21\) and in T-cell lines\(^6\) derived from patients with systemic sclerosis compared with healthy controls. It also has been reported that experimentally activated B cells might be prone to produce IL-6.\(^{22,23}\) Other lines of evidence implicate dermal fibroblasts as an important source of IL-6.\(^{13,24–28}\) In this report, although the expression of IL-6 mRNA in both lesional skin and draining LNs was increased by BLM treatment, the specific cell type producing IL-6 was not identified. Further studies are required to clarify the source(s) of IL-6.

How does secreted IL-6 contribute to the pathogenesis of scleroderma? IL-6 might modulate \(\alpha\)-SMA expression in dermal fibroblasts and induce myofibroblasts, which are known to produce collagen\(^{29}\) and induce sclerotic change.\(^{11,30}\) We observed IL-6 effects on \(\alpha\)-SMA expression from \(\alpha\)-KO dermal fibroblasts \textit{in vitro} in this study (Figure 4A). Unexpectedly, nontreated cultured wild-type dermal fibroblasts strongly expressed \(\alpha\)-SMA (Figure 4A), the expression of which was not affected by exogenous IL-6, whereas MR16-1 treatment decreased the expression of \(\alpha\)-SMA mRNA (Figure 4, A and B). Therefore, continuous autocrine production of IL-6 by wild-type cultured dermal fibroblasts might increase the threshold for reactivity to IL-6.

Furthermore, in both prevention and treatment protocols with MR16-1, the reduction in dermal sclerosis was accompanied by decreasing numbers of myofibroblasts, which are known as activated fibroblasts with strong fibrogenic property. The absence of myofibroblasts at the BLM injection site of \(\alpha\)-KO mice indicates that IL-6-induced dermal sclerosis occurs via induction of myofibroblasts. Thus, we hypothesize that MR16-1 and tocilizumab have favorable effects on scleroderma via prevention of fibroblast activation. Administration of tocili-
zumab to scleroderma patients exhibited ameliorating effects of skin sclerosis, and seemed to reduce the number of Erk-activated α-SMA-positive fibroblast in lesional skin (Figure 4D). These findings were inconclusive because of the number of cases, and further studies were required.

Another finding was reduction of LN swelling by MR16-1 treatment in mice in the BLM-induced model of scleroderma. We could not determine whether the LN swelling associated with BLM treatment was a cause or effect of BLM-induced skin sclerosis. Examination of the differential ratios of leukocytes, such as T cells, B cells, and macrophages, did not give any insight, as these were not altered after 4 weeks of BLM injection (data not shown). However, there was a slight, but significant, increase in the numbers of cells double-positive for PDCA-1+CD11c+ (Figure 6D) or B220+CD11c+ (data not shown) in the draining LNs of MR16-1–treated mice relative to control Ab–treated mice in the prevention model. This suggests that IL-6 might affect pDC numbers in the LNs. LN swelling is not a well-known symptom in scleroderma, and only a few articles describe LN findings in scleroderma. We should keep an eye on such symptoms.

Recent studies have indicated that pDCs may promote scleroderma via secretion of type 1 interferon, and in- tense antibody. However, other data suggest MHC class II–restricted proliferation of CD4+ T cells had been previously thought to contribute to the pathogenesis of scleroderma, one could speculate that an increased ratio of pDCs might prevent skin sclerosis via regulating peripheral tolerance. However, it is clear that the function of pDCs in pathogenesis of scleroderma is complex and needs further study.

The clear positive effects of IL-6 inhibition in mouse models with scleroderma indicate that further study of IL-6–secreting cells, effectors, and signaling in scleroderma holds great promise for the development of therapies for scleroderma, as well as for other diseases in which IL-6 can play a pivotal role.

Acknowledgments

We thank Prof. Junji Takeda (Osaka University) for expert comments and Dr. Toshiaki Hanafusa, Kenju Nishida, and Han Fu for technical assistance.

References


