

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

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**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  $\alpha$ -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  $\alpha$ -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)**

Patients with scleroderma frequently experience broad area skin sclerosis and internal organ involvement including pulmonary fibrosis, esophageal dysfunction, pulmonary arterial hypertension, renal crisis, and heart failure.<sup>1</sup> These symptoms dramatically affect the prognosis for scleroderma patients. Autoaggressive immunological activation and continuous activation of fibroblasts are the key components of scleroderma, yet the mechanisms underlying these are incompletely understood.

Several lines of evidence indicate that interleukin (IL)-6 contributes to the disease process in scleroderma. Serum IL-2, IL-4, IL-6, and tumor necrosis factor  $\alpha$  levels are elevated in scleroderma.<sup>2–6</sup> Increased serum IL-6 levels are also observed in both scleroderma mouse models: the bleomycin (BLM)-induced scleroderma mouse and the type 1 tight-skin mouse (*Tsk1/+*).<sup>7,8</sup> At the cellular level, IL-6–producing T helper type 2 clones contribute to anti-DNA topoisomerase I autoantibody, a key autoantibody in scleroderma.<sup>9</sup> The point of action of IL-6 in scleroderma remains controversial, with some evidence suggesting the final maturation step of B cells<sup>10</sup> and/or activation of fibroblasts.<sup>11–13</sup> Thus, although the specific function of IL-6 in the pathogenesis of scleroderma remains unclear, there is ample evidence that inhibition of IL-6–mediated signaling might be a route to better treatment for scleroderma.

In this study, we used the BLM-induced scleroderma mouse model to demonstrate the importance of IL-6 in pathogenesis of scleroderma and to evaluate the effect of anti-mouse IL-6 receptor monoclonal antibody. We have examined whether MR16-1 acts directly on dermal fibroblasts by investigating the induction of myofibroblasts *in vitro*. We also examined the tissue of scleroderma patients treated with tocilizumab, a humanized monoclonal antibody (Ab) against the IL-6 receptor used to treat rheumatoid arthritis or Castleman's disease.<sup>14</sup> Patients

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with intractable scleroderma treated with tocilizumab were previously reported to show marked amelioration of dermal sclerosis.<sup>15</sup> 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.<sup>4,16</sup> 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% CO<sub>2</sub>.

The primary *Il-6*KO 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.<sup>15</sup>

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-6*KO 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- $\alpha$ -smooth muscle actin Ab (1:100,  $\alpha$ -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  $\alpha$ -SMA (Invitrogen, Carlsbad, CA) and Hoechst 33342 (Molecular Probes, Eugene, OR). Mouse IgG<sub>2a</sub> (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 PBST, they were double-stained with mouse monoclonal anti- $\alpha$ -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  $\alpha$ -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

	Thickness (mm)			Hardness (Arbitrary)		
	1st (n = 4)	2nd (n = 4)	3rd (n = 4)	1st (n = 4)	2nd (n = 4)	3rd (n = 4)
PBS						
Control Ab	0.137 ± 0.016	0.10 ± 0.01	0.123 ± 0.010	7.09 ± 2.17	6.14 ± 0.72	4.86 ± 1.05
MR16-1	0.11 ± 0.01	0.111 ± 0.011	0.122 ± 0.013	8.91 ± 2.59	6.45 ± 1.49	4.92 ± 0.77
% Change						
Each experiment	81.82	106.33	98.65	125.64	104.91	101.15
Mean ± SE		95.60 ± 7.238			110.6 ± 7.614	
BLM						
Control Ab	0.33 ± 0.04*	0.36 ± 0.05*	0.30 ± 0.07*	24.14 ± 5.58*	16.61 ± 1.90*	9.93 ± 1.51*
MR16-1	0.22 ± 0.03 <sup>††</sup>	0.29 ± 0.02 <sup>‡§</sup>	0.16 ± 0.03 <sup>†</sup>	15.47 ± 4.52	8.53 ± 0.80 <sup>†</sup>	5.92 ± 0.49 <sup>†</sup>
% Change						
Each experiment	67.09	81.29	52.22	64.08	51.33	59.59
Mean ± SE		66.89 ± 8.39			58.34 ± 3.74	

(table continues)

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 (%).

\**P* < 0.01 PBS+Control Ab versus BLM+Control Ab.

<sup>†</sup>*P* < 0.01 BLM+Control Ab versus BLM+MR16-1.

<sup>‡</sup>*P* < 0.01 PBS+MR16-1 versus BLM+MR16-1.

<sup>§</sup>*P* < 0.05 BLM+Control Ab versus BLM+MR16-1.

HPS, high-power field.

### Western Blot Analysis

Wild-type and *Il-6*KO fibroblasts were prepared as described above and cultured to semiconfluence in 100-cm<sup>2</sup> 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  $\mu$ mol/L 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 rinsed twice with ice-cold PBS, and total cell protein was collected in 500  $\mu$ L 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.<sup>4</sup> 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), 150 mmol/L NaCl, and 0.1% v/v Tween-20]. The membranes were incubated with mouse monoclonal anti- $\alpha$ -SMA (Dako-Cytomation) Ab at a dilution of 1:1000 overnight at 4°C or with mouse monoclonal anti- $\beta$ -actin (Sigma-Aldrich) at a dilution of 1:5000 for 30 minutes at room temperature. After three 5-minute washes in TBST, membranes were incubated with horseradish peroxidase-conjugated anti-mouse 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<sup>17</sup> 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  $\mu$ L) 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.<sup>7</sup> Control mice received 100  $\mu$ L 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'-ACACACTGGTCTGAGGGAC-3', antisense 5'-TACCACAAGGTTGGCAGGTG-3'; GAPDH,

**Table 1.** Continued

$\alpha$ -SMA-Positive Cells (Cells/HPS)			Mast Cells (Cells/HPS)		
1st (n = 4)	2nd (n = 4)	3rd (n = 4)	1st (n = 4)	2nd (n = 4)	3rd (n = 4)
6.00 $\pm$ 4.08	3.50 $\pm$ 1.29	8.75 $\pm$ 1.50	12.50 $\pm$ 4.51	21.67 $\pm$ 3.06	31.50 $\pm$ 5.45
5.00 $\pm$ 1.73	4.00 $\pm$ 1.41	10.00 $\pm$ 4.16	11.00 $\pm$ 1.83	17.33 $\pm$ 6.11	29.75 $\pm$ 3.50
83.33	114.29	114.29	88.00	80.00	94.44
	104.0 $\pm$ 10.32			87.48 $\pm$ 4.177	
14.00 $\pm$ 1.83*	14.50 $\pm$ 4.93*	27.75 $\pm$ 0.96*	46.50 $\pm$ 8.43*	35.33 $\pm$ 5.69*	67.25 $\pm$ 5.85*
10.00 $\pm$ 1.41	7.00 $\pm$ 1.82 <sup>§</sup>	15.25 $\pm$ 2.75 <sup>†</sup>	20.25 $\pm$ 4.99 <sup>†</sup>	25.00 $\pm$ 2.00	29.50 $\pm$ 6.19 <sup>†</sup>
71.43	48.28	54.95	43.55	70.75	43.87
	58.22 $\pm$ 6.88			52.72 $\pm$ 9.02	

**Table 2.** Effect of MR16-1 on BLM-Induced Dermal Sclerosis in a Treatment Model

	Thickness (mm)		Hardness (arbitrary)		$\alpha$ -SMA-positive cells (cells/HPS)		Mast cells (cells/HPS)	
	1st (n = 4)	2nd (n = 3)	1st (n = 4)	2nd (n = 3)	1st (n = 4)	2nd (n = 3)	1st (n = 4)	2nd (n = 3)
PBS								
Control Ab	0.14 $\pm$ 0.02	0.12 $\pm$ 0.03	4.80 $\pm$ 0.47	6.19 $\pm$ 1.29	3.75 $\pm$ 1.50	2.33 $\pm$ 0.58	13.50 $\pm$ 1.73	19.67 $\pm$ 2.52
MR16-1	0.12 $\pm$ 0.01	0.107 $\pm$ 0.006	4.67 $\pm$ 0.47	5.68 $\pm$ 0.43	3.33 $\pm$ 0.56	2.67 $\pm$ 1.15	15.33 $\pm$ 1.15	21.00 $\pm$ 2.00
% Changes	83.64	86.49	97.22	91.78	88.88	106.78	113.58	106.78
BLM								
Control Ab	0.30 $\pm$ 0.03*	0.29 $\pm$ 0.03*	9.34 $\pm$ 1.58*	9.81 $\pm$ 1.17 <sup>†</sup>	10.00 $\pm$ 2.58*	7.67 $\pm$ 0.58*	37.75 $\pm$ 2.50*	54.67 $\pm$ 8.39*
MR16-1	0.21 $\pm$ 0.05 <sup>‡</sup>	0.18 $\pm$ 0.03 <sup>§</sup>	5.46 $\pm$ 0.62 <sup>¶</sup>	5.41 $\pm$ 0.77 <sup>¶</sup>	5.50 $\pm$ 1.29 <sup>¶</sup>	4.00 $\pm$ 1.00 <sup>¶</sup>	27.00 $\pm$ 2.16 <sup>¶</sup>	37.00 $\pm$ 6.56 <sup>¶</sup>
% Changes	70.00	62.50	58.46	55.12	55.00	52.17	71.52	67.68

Mean  $\pm$  SD is presented. To quantify the impact of MR16-1 treatment, % changes were calculated as follows: (evaluative consequences of MR16-1 treatment/that of PBS treatment)  $\times$  100 (%).

\* $P < 0.01$  PBS+Control Ab versus BLM+Cont. Ab.

<sup>†</sup> $P < 0.05$  PBS+Control Ab versus BLM+Cont. Ab.

<sup>‡</sup> $P < 0.05$  BLM+Control Ab versus BLM+MR16-1.

<sup>§</sup> $P < 0.01$  PBS+MR16-1 versus BLM+MR16-1.

<sup>¶</sup> $P < 0.01$  BLM+Control Ab versus BLM+MR16-1.

<sup>||</sup> $P < 0.05$  PBS+MR16-1 versus BLM+MR16-1.

HPS, high-power field.

sense 5'-TGTCATCATACTTGGCAGGTTTCT-3', anti-sense 5'-CATGGCCTTCCGTGTTCTTA-3'. Real-time PCR (40 cycles of denaturing at 92°C for 15 seconds and annealing at 60°C for 60 seconds) was run on an ABI 7000 Prism Detection System (Applied Biosystems).

### Mouse IL-6 Receptor-Specific Monoclonal Antibody Treatment

Rat anti-mouse IL-6 receptor monoclonal Ab (clone MR16-1, rat IgG<sub>1</sub>) described previously<sup>18</sup> was provided by Chugai Pharmaceutical (Shizuoka, Japan). Purified rat IgG<sub>1</sub> (isotype-matched control Ab) (Cappel, MP Biomedicals, Solon, OH) was administered as a control. Preventive and therapeutic administration methods are discussed later. Percentage to control values were calculated as follows: (mean actual value/mean control value)  $\times$  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-6*KO 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.<sup>19</sup> 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

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

	Thickness (mm)		Hardness (arbitrary)		$\alpha$ -SMA-positive cells (cells/HPS)		Mast cells (cells/HPS)	
	1st (n = 4)	2nd (n = 3)	1st (n = 4)	2nd (n = 3)	1st (n = 4)	2nd (n = 3)	1st (n = 4)	2nd (n = 3)
WT								
PBS	0.112 $\pm$ 0.013	0.14 $\pm$ 0.04	4.91 $\pm$ 0.38	5.46 $\pm$ 0.93	4.75 $\pm$ 1.50	8.00 $\pm$ 2.00	18.00 $\pm$ 3.16	28.33 $\pm$ 4.51
BLM	0.32 $\pm$ 0.02*	0.29 $\pm$ 0.06 <sup>†</sup>	12.38 $\pm$ 0.81*	9.69 $\pm$ 0.51*	19.75 $\pm$ 5.74*	23.33 $\pm$ 6.11*	40.25 $\pm$ 2.22*	63.33 $\pm$ 9.87*
% Change	282.85	203.13	251.99	177.28	415.79	291.67	223.61	223.53
<i>Il-6</i>								
PBS	0.111 $\pm$ 0.010	0.12 $\pm$ 0.01	5.37 $\pm$ 0.48	5.12 $\pm$ 0.71	4.00 $\pm$ 1.41	6.67 $\pm$ 2.52	17.75 $\pm$ 2.5	29.5 $\pm$ 0.71
BLM	0.22 $\pm$ 0.03 <sup>‡§</sup>	0.17 $\pm$ 0.03 <sup>‡</sup>	7.14 $\pm$ 0.96 <sup>¶  </sup>	5.85 $\pm$ 0.21 <sup>‡</sup>	9.50 $\pm$ 4.43 <sup>  </sup>	13.00 $\pm$ 1.73 <sup>  </sup>	22.75 $\pm$ 4.79 <sup>‡</sup>	34.00 $\pm$ 7.00 <sup>  </sup>
% Change	195.71	133.81	132.87	114.21	237.50	195.00	128.17	115.25

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

\* $P < 0.01$  WT with PBS versus WT with BLM.

<sup>†</sup> $P < 0.05$  WT with PBS versus WT with BLM.

<sup>‡</sup> $P < 0.01$  WT with BLM versus *Il-6*KO with BLM.

<sup>§</sup> $P < 0.01$  *Il-6*KO with PBS versus *Il-6*KO with BLM.

<sup>¶</sup> $P < 0.05$  *Il-6*KO with PBS versus *Il-6*KO with BLM.

<sup>||</sup> $P < 0.05$  WT with BLM versus *Il-6*KO with BLM.

HPS, high-power field; WT, wild-type.

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

	1st experiment (total number/lymph node, $\times 10^7$ )			2nd experiment (total number/lymph node, $\times 10^6$ )		
	PBS	BLM	% Change	PBS	BLM	% Change
WT						
Control Ab	$0.97 \pm 0.21$ ( $n = 3$ )	$2.17 \pm 0.31^*$ ( $n = 3$ )	222.61	$1.90 \pm 0.41$ ( $n = 4$ )	$3.55 \pm 0.21^*$ ( $n = 4$ )	186.73
MR16-1	$0.76 \pm 0.02$ ( $n = 3$ )	$0.80 \pm 0.12^\dagger$ ( $n = 3$ )	105.29	$1.85 \pm 0.18$ ( $n = 4$ )	$1.85 \pm 0.34^\dagger$ ( $n = 4$ )	99.84
IL-6KO						
WT	$0.81 \pm 0.18$ ( $n = 3$ )	$1.53 \pm 0.40^\ddagger$ ( $n = 3$ )	190.03	$2.36 \pm 0.26$ ( $n = 3$ )	$3.71 \pm 0.61^\ddagger$ ( $n = 3$ )	157.42
IL-6KO	$0.66 \pm 0.26$ ( $n = 3$ )	$0.54 \pm 0.09^\S$ ( $n = 3$ )	82.73	$1.97 \pm 0.67$ ( $n = 3$ )	$2.20 \pm 0.29^\S$ ( $n = 3$ )	111.51

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

\* $P < 0.01$  PBS+Control Ab versus BLM+Cont. Ab.

$^\dagger P < 0.01$  BLM+Control Ab versus BLM+MR16-1.

$^\ddagger P < 0.05$  PBS+Control Ab versus BLM+Cont. Ab.

$^\S P < 0.05$  BLM+Control Ab versus BLM+MR16-1.

probe divided by the pressure of the indenter in a connected computer.

### 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- $\mu$ m thickness using a microtome. Sections were stained with hematoxylin and eosin (H&E). Dermal thickness (measured from the epidermal-dermal junction to dermal-fat junction) was determined at  $\times 100$  magnification at three randomly selected sites in each animal. Mast cells were identified in 3- $\mu$ m deparaffinized sections stained with 1% Toluidine Blue, and mast cells were counted in 10 randomly selected sites under  $\times 400$  power using light microscopy.

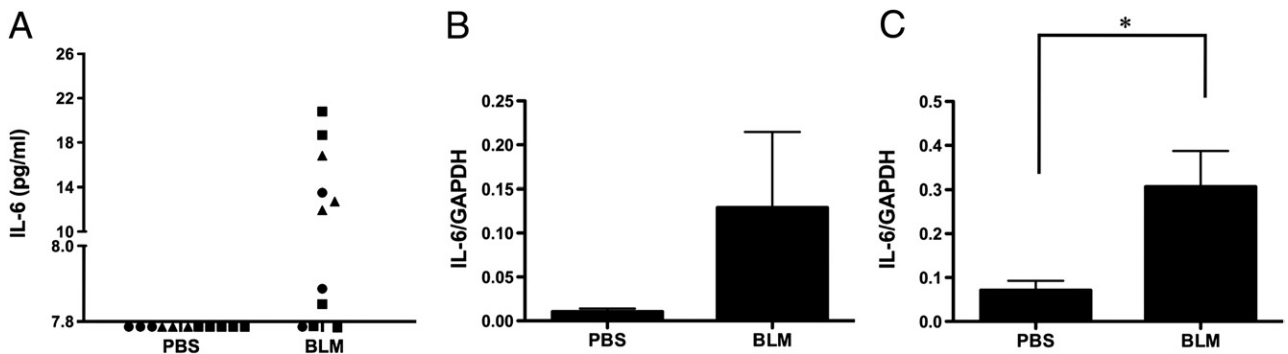
### Immunohistochemical Analysis of $\alpha$ -SMA

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

nols. Next, endogenous peroxide was blocked using 3%  $H_2O_2$  in methanol for 5 minutes. Slides were blocked with 2% bovine serum albumin for 10 minutes, and stained with primary Ab (anti- $\alpha$ -SMA Ab 1:100 dilution) for 60 minutes. After washing with PBS containing 0.05% Triton, they were developed using Dako ChemMate Envision Kit/horseradish peroxidase (Dako-Cytomation) for 30 minutes, and counterstained with hematoxylin.  $\alpha$ -SMA-positive fibroblastic cells were counted in 10 randomly selected sites under  $\times 400$  power using light microscopy.

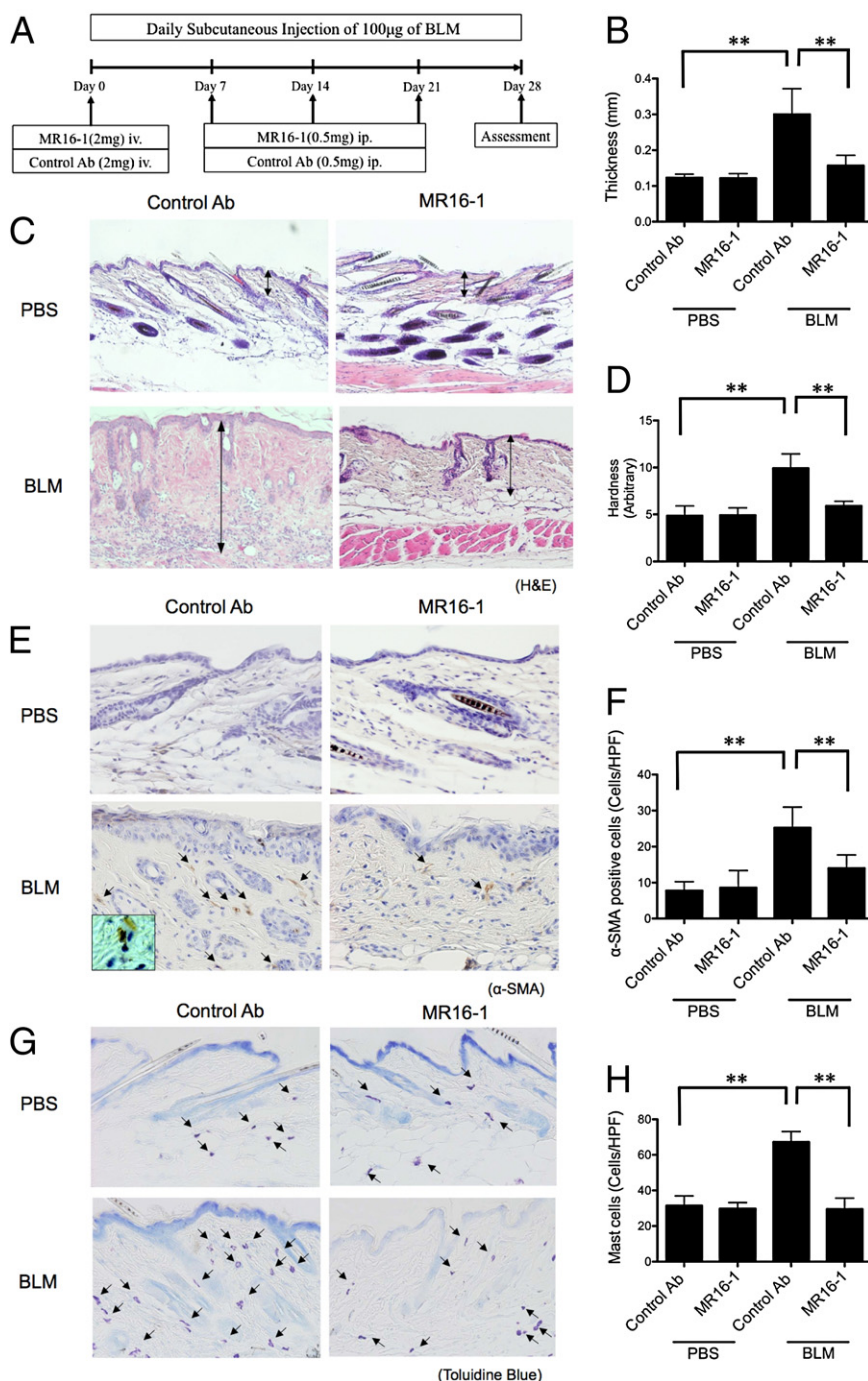
### Flow Cytometric Analysis

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).



**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  $\mu$ L/day,  $n = 11$ ) or PBS (100  $\mu$ L/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 [box ( $n = 5$ ), circle ( $n = 3$ ), and triangles ( $n = 3$ )]. Each symbol represents one IL-6 measurement for a single mouse, and symbols below 7.8 pg/mL indicate mice for which IL-6 was below the limit of detection. enzyme-linked immunosorbent assays were run in duplicate for all mice, with similar results. **B and C:** Expression of IL-6 mRNA was measured by real-time PCR. RNA was extracted from skin lesions (**B**) and cutaneous draining LNs (**C**) from C57BL/6 mice treated with PBS ( $n = 3$ ) or BLM ( $n = 3$ ) for 4 weeks. Data were normalized to the GAPDH internal control. Bars represent mean  $\pm$  SD. \* $P < 0.05$ , unpaired  $t$ -test. Data in B and C are from one of two independent experiments that gave similar results. The IL-6/GAPDH data (mean  $\pm$  SD) for skin lesions (**B**) were as follows: first experiment ( $n = 3$ ), PBS:  $0.011 \pm 0.006$ , BLM:  $0.129 \pm 0.148$  ( $P = 0.2391$ ); second experiment ( $n = 3$ ), PBS:  $0.142 \pm 0.070$ , BLM:  $0.441 \pm 0.283$  ( $P = 0.1495$ ). The IL-6/GAPDH data (mean  $\pm$  SD) for cutaneous draining LNs (**C**) were as follows: first experiment ( $n = 3$ ), PBS:  $0.071 \pm 0.038$ , BLM:  $0.306 \pm 0.141$  ( $P = 0.0493$ ), second experiment ( $n = 3$ ), PBS:  $0.083 \pm 0.067$ , BLM:  $0.430 \pm 0.175$  ( $P = 0.0321$ ).





**Figure 2.** Effect of MR16-1 on BLM-induced dermal sclerosis in a prevention model. **A:** Experimental protocol for prevention of BLM-induced dermal sclerosis by administration of MR16-1 or control Ab to either PBS- or BLM-treated mice ( $n = 4$  for each group). **B:** Measurements of dermal thickness ( $n = 4$  for each group). **C:** H&E staining of specimens derived from PBS- or BLM-injected mice treated with MR16-1 or control Ab (original magnification,  $\times 40$ ). The length of each **two-headed arrow** indicates the measurement region of dermal thickness. **D:** Skin hardness measurements obtained using a Vesmeter ( $n = 4$  for each group). **E:** Immunohistochemical staining for  $\alpha$ -SMA. **Arrows** indicate  $\alpha$ -SMA-positive fibroblasts (original magnification,  $\times 100$ ). **Inset** photo shows higher magnification ( $\times 200$ ) of  $\alpha$ -SMA-positive fibroblasts. **F:** The number of  $\alpha$ -SMA-positive fibroblasts per high-power field (HPF,  $\times 400$ ) was 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. **G:** Results of Toluidine Blue staining. **Arrows** indicate the metachromatically stained mast cells (original magnification,  $\times 100$ ). **H:** The number of mast cells per HPF ( $\times 400$ ) was 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, D, F, and H:** 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 third of three independent experiments with similar results presented in Table 1.

## Computation Methods and Statistical Analysis

All data except change ratios are expressed as mean values  $\pm$  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  $\pm$  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 de-

termine 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

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

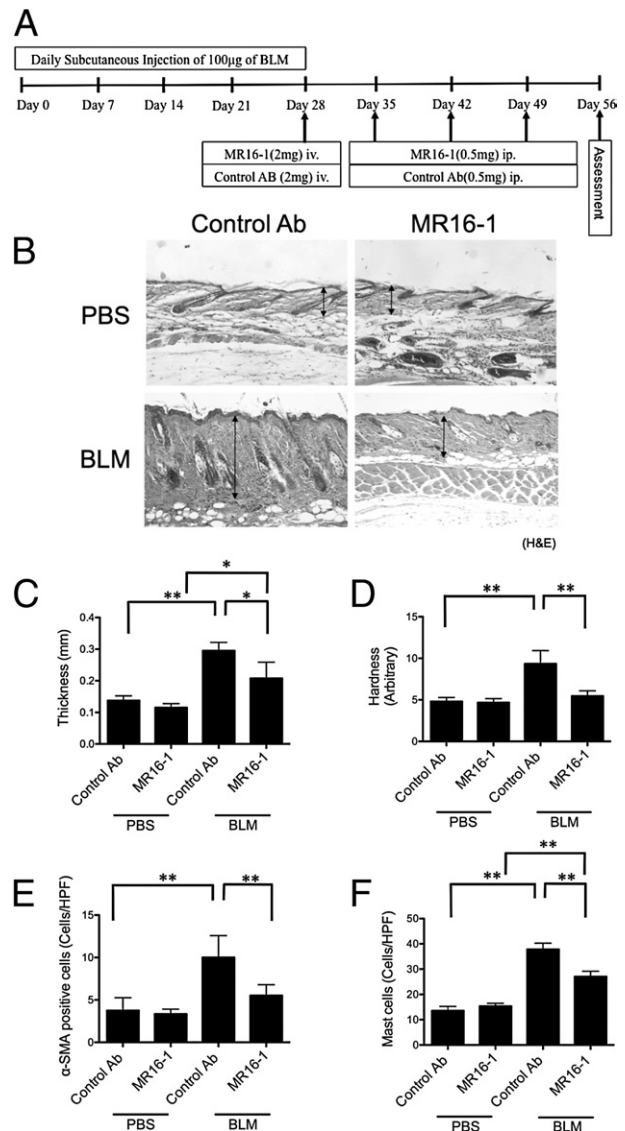
We next investigated whether MR16-1, a rat anti-mouse IL-6 receptor monoclonal Ab, could ameliorate the dermal thickening and skin hardening symptoms observed in BLM-treated mice. Figure 2A shows the administration schedule of preventive intervention. Dermal thickness was significantly increased at the 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 summarizes the data from two treatment intervention experiments. These results indicate that IL-6 may contribute to the pathogenesis of BLM-

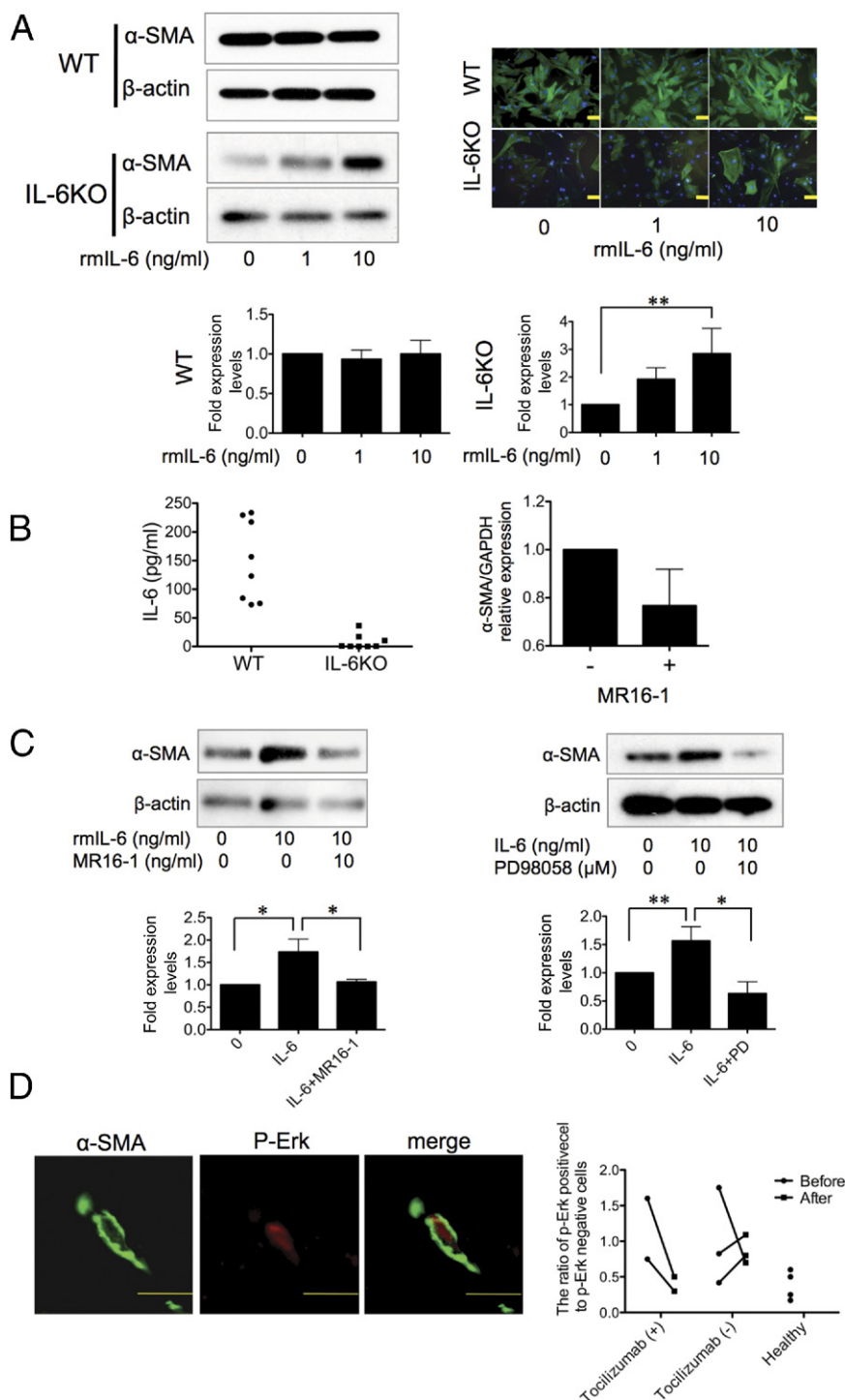


**Figure 3.** Effect of MR16-1 on BLM-induced dermal sclerosis in a treatment model. **A:** Experimental protocol for treatment of BLM-induced dermal sclerosis by administration of MR16-1 or control Ab to either PBS- or BLM-treated mice ( $n = 3-4$  for each group). The effect of Ab therapy was assessed 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.

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 4.** IL-6 induces  $\alpha$ -SMA protein expression in cultured IL-6KO fibroblasts. **A:**  $\alpha$ -SMA expression following recombinant mouse IL-6 (rmIL-6) stimulation was determined by immunofluorescent staining and Western blot analysis.  $\alpha$ -SMA and nucleus were shown in green and blue, respectively. Scale bar = 100  $\mu$ m.  $\beta$ -Actin expression was used to determine fold changes in expression by densitometry. Cultured dermal fibroblasts from wild-type (WT) and IL-6KO mice were treated with 0, 1, and 10 ng/mL rmIL-6 for 24 hours. These experiments were repeated three times, and the results of densitometric analyses are presented as the fold change (mean  $\pm$  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-6KO mice (**left**) after 24 hours. MRI6-1 treatment decreased  $\alpha$ -SMA mRNA expression in cultured primary WT dermal fibroblasts (**right**). **C:** MRI6-1 and ERK inhibitor, PD98058, attenuated rmIL-6-induced  $\alpha$ -SMA protein expression in cultured IL-6KO fibroblasts.  $\beta$ -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  $\pm$  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  $\alpha$ -SMA (green) in lesional skin derived from two tocilizumab-treated patients with scleroderma. A representative image of p-ERK<sup>+</sup>,  $\alpha$ -SMA<sup>+</sup> fibroblasts (original magnification,  $\times$ 1200) is shown. The number of p-ERK-positive  $\alpha$ -SMA-positive fibroblasts per HPF ( $\times$ 400) was determined by observation of 10 random grids. Scale bar = 50  $\mu$ m. The ratio of p-ERK-positive fibroblasts was calculated as follows: the number of p-ERK<sup>+</sup>,  $\alpha$ -SMA<sup>+</sup> fibroblasts/the number of p-ERK<sup>+</sup>,  $\alpha$ -SMA<sup>+</sup> fibroblasts.

with exogenous recombinant mouse IL-6 (rmIL-6) did not alter  $\alpha$ -SMA expression (Figure 4A). Further, highly expressed levels of endogenous IL-6 from nontreated cultured primary wild-type dermal fibroblasts and decreased levels of  $\alpha$ -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  $\alpha$ -SMA by IL-6 (Figure 4B). Thus, we switched to primary *Il-6*KO mouse-derived fibroblasts and evaluated  $\alpha$ -SMA expres-

sion 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  $\alpha$ -SMA expression in a dose-dependent manner (Figure 4A).  $\alpha$ -SMA induction by rmIL-6 was inhibited by 10 ng/mL MR16-1 and also by the ERK1/2 inhibitor PD98059 (Figure 4C).

These results led us to examine whether the positive effects of clinical treatment with tocilizumab might correlate with reduced numbers of ERK-activated  $\alpha$ -SMA-pos-



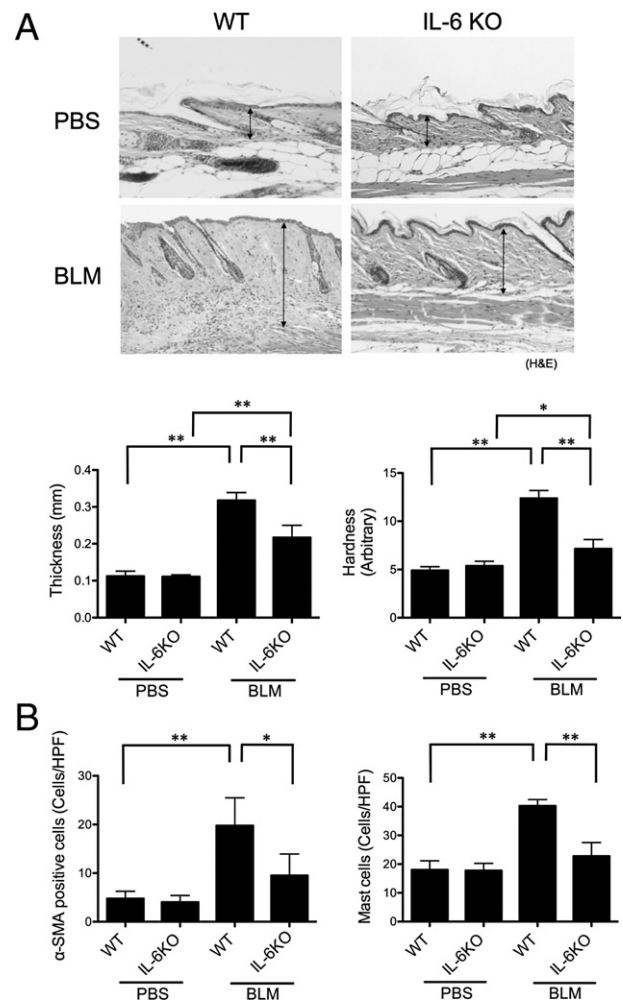
itive dermal fibroblasts in lesional skin of scleroderma patients. The number of Erk-activated  $\alpha$ -SMA-positive cells in lesional skin in scleroderma patients treated with tocilizumab for 6 months was reduced to a similar level as in healthy skin, whereas the number in scleroderma patients treated 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-6*KO Mice

To investigate the role of IL-6 in BLM-induced dermal sclerosis, *Il-6*KO 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-6*KO mice was attenuated compared with that in wild-type mice. Lack of visible changes in the skin between PBS-treated *Il-6*KO 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-6*KO mice were significantly attenuated compared to wild-type mice (Figure 5A). The numbers of  $\alpha$ -SMA-positive cells and mast cells in BLM-treated *Il-6*KO 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<sup>+</sup>CD11c<sup>+</sup> 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-6*KO mice (Figure 6C, Table 4), consistent with weight and total cell count per LN measurements in the normal range (Figure 6C).



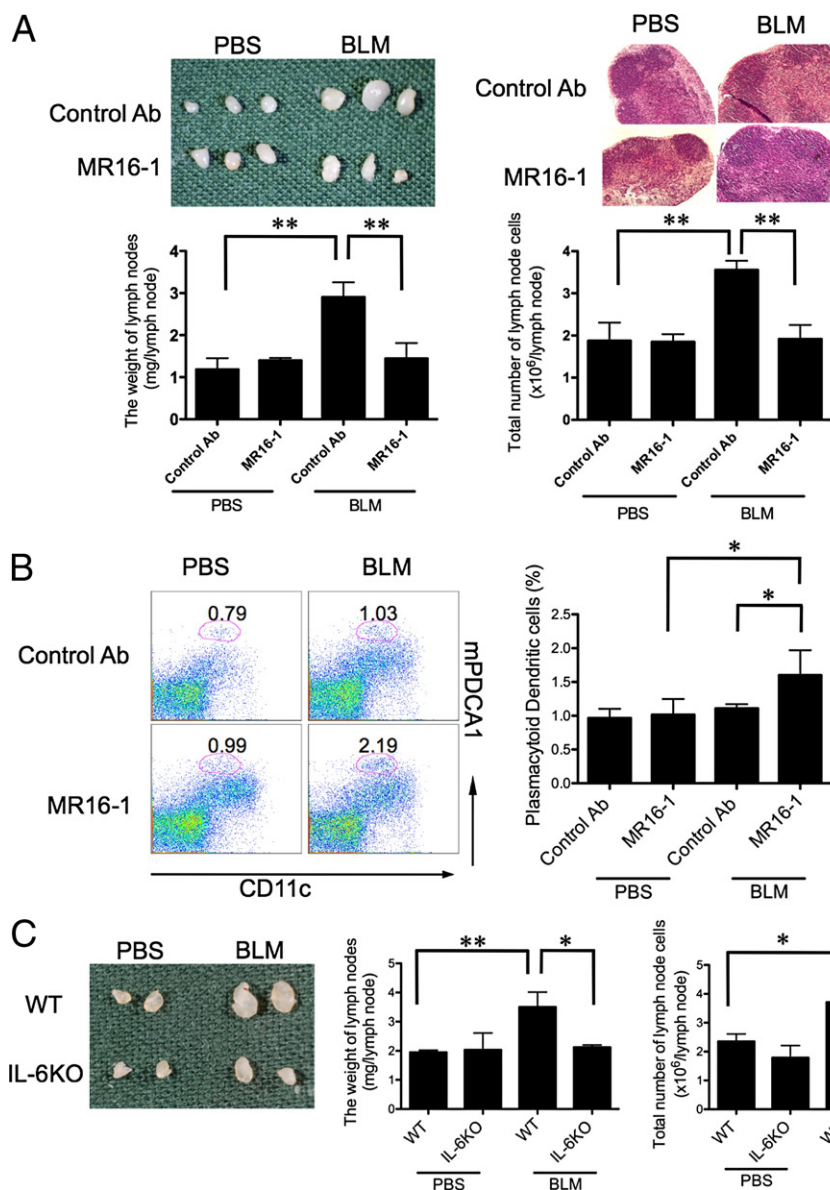
**Figure 5.** Attenuated BLM-induced dermal sclerosis in *Il-6*KO mice. **A:** H&E staining of skin specimen derived from PBS- and BLM-treated wild-type (WT) and *Il-6*KO mice (original magnification  $\times 40$ ), and measurements of dermal thickness (lower left panel) and skin hardness (lower right panel). The length of the two-headed arrows indicates the measurement region of dermal thickness. **B:** The number of  $\alpha$ -SMA-positive fibroblasts (left panel) and mast cells (right panel) per HPF ( $\times 400$ ) was determined by observation of 10 random grids. **A** and **B:** Bars represent mean  $\pm$  SD ( $n = 4$  for each group). \* $P < 0.05$ , \*\* $P < 0.01$ , one-way analysis of variance and Bonferroni post hoc multiple comparison. Data presented are from the first of two independent experiments that yielded similar results, and Table 3 presents data from both experiments.

We then examined whether LNs were enlarged in a patient with scleroderma, and found swelling of axillary LNs on computed tomography scan (Figure 6D), which was not detected after the administration of tocilizumab (Figure 6D).

### 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



**Figure 6.** The size of enlarged draining LNs was reduced by administration of MR16-1 in the model mouse and in a patient with scleroderma. **A:** Gross appearance and H&E staining of cutaneous draining LNs in a prevention model. The weight per LN and total cell count per LN were measured ( $n = 4$ ). **B:** Cell surface marker staining of lymphocytes on cutaneous draining LNs in a prevention model. Staining results for PDCA-1 and CD11c are shown. Gated area indicates fraction of pDCs, and the value inside the dot plot is the percentage of pDC fraction. Flow cytometric analysis was performed on pooled cutaneous draining LNs from four mice per group. **C:** Gross appearance of cutaneous draining LNs (**left panel**) derived from a PBS-treated wild-type (WT) mouse, a BLM-treated WT mouse, a PBS-treated *IL-6*KO mouse, and a BLM-treated *IL-6*KO mouse. The weight per LN (**center panel**) and total cell count per LN (**right panel**) were measured ( $n = 3$  for each group). **D:** Computed tomography scan with arrows indicating enlarged axillary LNs of the patient with scleroderma before and after administration of tocilizumab for 6 months. \* $P < 0.05$ , \*\* $P < 0.01$ , one-way analysis of variance and Bonferroni post hoc multiple comparison. The data presented are from the second of two independent experiments that yielded similar results (see Table 4 for data from both experiments), except for the evaluation of weight of LNs, which was performed in only one of those experiments.

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<sup>6,20,21</sup> and in T-cell lines<sup>6</sup> 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.<sup>22,23</sup> Other lines of evidence implicate dermal fibroblasts as an important source of IL-6.<sup>13,24–28</sup> 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 myfibroblasts, which are known to produce collagen<sup>29</sup> and induce sclerotic change.<sup>11,30</sup> We observed IL-6 effects on  $\alpha$ -SMA expres-

sion from *IL-6*KO dermal fibroblasts *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 myfibroblasts, which are known as activated fibroblasts with strong fibrogenic property. The absence of myfibroblasts at the BLM injection site of *IL-6*KO mice indicates that IL-6–induced dermal sclerosis occurs via induction of myfibroblasts. 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,<sup>15</sup> and seemed to reduce the number of Erk-activated  $\alpha$ -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<sup>+</sup>CD11c<sup>+</sup> (Figure 6D) or B220<sup>+</sup>CD11c<sup>+</sup> (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.<sup>31</sup> We should keep an eye on such symptoms.

Recent studies have indicated that pDCs may promote scleroderma via secretion of type 1 interferon,<sup>32</sup> and induction of type 1 interferon was found by anti-topoisomerase antibody-containing serum, but not by anti-centromere antibody.<sup>32,33</sup> However, other data suggest MHC class II-restricted antigen presentation by pDCs might inhibit T-cell-mediated autoimmunity via selective expansion of Ag-specific natural regulatory T cells.<sup>34</sup> Because MHC class II-restricted proliferation of CD4<sup>+</sup> T cells had been previously thought to contribute to the pathogenesis of scleroderma,<sup>35</sup> 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.

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