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Short Communication

Mesenchymal Stem Cells Stably Transduced with a Dominant-Negative Inhibitor of CCL2 Greatly Attenuate Bleomycin-Induced Lung Damage

Shigeki Saito,* Takayuki Nakayama,* Naozumi Hashimoto,† Yasuhiko Miyata,‡ Kensuke Egashira,§ Norihiko Nakao,* Satoshi Nishiwaki,* Minoru Hasegawa,¶ Yoshinori Hasegawa,† and Tomoki Naoe*

From the Departments of Hematology and Oncology and Respiratory Medicine,† Nagoya University Graduate School of Medicine, Nagoya; the Department of Hematology,‡ Nagoya Medical Center, Nagoya; the Department of Cardiovascular Medicine,§ Kyushu University Graduate School of Medicine, Fukuoka; and the Department of Dermatology,¶ Kanazawa University Graduate School of Medical Science, Ishikawa, Japan*

Acute respiratory distress syndrome (ARDS) is a crippling disease with no effective therapy characterized by progressive dyspnea. Mesenchymal stem cells (MSCs) have emerged as a new therapeutic modality for ARDS because MSCs can attenuate inflammation and repair the damaged tissue by differentiating into several cell types. Macrophages participate in the development of ARDS; however, MSCs only weakly modulate macrophage function. The chemokine CCL2 is a potent inducer of macrophage recruitment and activation, and its expression is elevated in patients with ARDS. We established MSCs that are stably transduced by a lentiviral vector expressing 7ND, a dominant-negative inhibitor of CCL2, to enhance the therapeutic function of MSCs. 7ND-MSCs retained the innate properties of MSCs and produced a large amount of 7ND. Many 7ND-MSCs were detected in bleomycin-treated lungs (immunostaining 24 hours after injection), suggesting that MSCs could work as a drug delivery tool. Mice treated with 7ND-MSCs showed significantly milder weight loss, lung injury, collagen content, accumulation of inflammatory cells and inflammatory mediators that were induced by bleomycin, and subsequent survival benefit. No evidence of 7ND-MSC–induced toxicity was observed during or after treatment. Thus, inhibiting the effects of macrophages may greatly enhance the ability of MSCs to effect lung repair in ARDS. ***(Am J Pathol 2011, 179:1088–1094; DOI: 10.1016/j.ajpath.2011.05.027)***

Acute respiratory distress syndrome (ARDS) is a severe inflammatory disorder characterized by diffuse pulmonary injury and subsequent fibrosis.[1] The development of ARDS is divided into two stages: an acute exudative phase and a proliferative phase. In the exudative phase, inflammatory cells, including macrophages, neutrophils, and lymphocytes, accumulate and secrete inflammatory mediators. After approximately 1 to 2 weeks, the exudative phase evolves into the proliferative phase, which is characterized by type II epithelial cell hyperplasia and accumulation of fibroblasts and numerous monocytes in the interstitium. Acinar architecture is destroyed and is replaced by thick fibrotic tissue. Once remodeling has occurred, fibrosis is irreversible and leads to pulmonary dysfunction.[2] Thus, the degree of fibrosis and the survival rate are inversely parallel,[3] suggesting that inflammatory cells and mediators are critical targets for ARDS.

Mesenchymal stem cells (MSCs) have emerged as a new therapeutic modality for ARDS by modulating immunoreactions and repairing damaged tissue. However, MSC effectiveness was limited in a bleomycin (BLM)-induced lung injury mouse model,[4,5] likely because MSCs can modulate T-cell, B-cell, natural killer cell, and dendritic cell function but cannot greatly modulate macrophage function.

CCL2 was first cloned as a proinflammatory CC chemokine for monocytes. Later, CCL2 was shown to recruit T cells, dendritic cells, and fibrocytes through binding to its receptor, CCR2.[6] CCL2 has been reported to be elevated in bronchoalveolar lavage (BAL) fluid, and this elevation is

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Address reprint requests to Takayuki Nakayama, M.D., Ph.D., Department of Hematology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan. E-mail: tnaka@med.nagoya-u.ac.jp.

closely related to disease severity in patients with ARDS.[7–9] A potent role of the CCL2-CCR2 axis in the development of lung fibrosis has also been demonstrated in genetically modified mice.[10–12] A deletion mutant of CCL2, 7ND, functions as a dominant-negative inhibitor of CCL2.[13,14]

MSCs are useful not only to modulate cell function but also as a vehicle for gene expression because MSCs accumulate at the site of lung injury.[4] We, therefore, hypothesized that a combination of MSCs and 7ND might synergistically ameliorate lung injury. Herein, we report that MSCs stably transduced with the *7ND* gene greatly attenuate BLM-induced lung damage in mice.

Materials and Methods

Animal Studies

Male 6- to 10-week-old C57BL/6J mice were purchased from Chubu Kagaku Shizai (Nagoya, Japan). The animal experiments were approved by the Institutional Ethics Committee for Laboratory Animal Research, Nagoya University School of Medicine, and were performed according to the guidelines of the institute.

Cells

MSCs were established from C57BL/6N mice as described elsewhere.[15] Cultures of passages 5 to 15 were used. A murine macrophage cell line, RAW264.7, was purchased from American Type Culture Collection (Manassas, VA) and was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS).

Plasmids, Vector Production, and in Vitro *Transduction*

The FLAG-tagged (3′ C terminus) deletion mutant CCL2 (7ND) was recloned from the 7ND pCDNA3 expression vector[16] into a lentiviral vector (pBGJR-EGFP; a gift from Dr. Stefano Rivella, Cornell University, New York, NY) by using unique NheI and XbaI sites. An empty pBGJR-EGFP vector was used as a control. We produced vector stocks by transient transfection of 293T cells using the envelope-encoding plasmid pLP/VSVG, the packaging plasmid pCMV-dR8.91, and pBGJR-EGFP-7ND or empty pBGJR-EGFP using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). MSCs were incubated with vector stocks in the presence of Polybrene, 4 μg/mL (Sigma-Aldrich, St. Louis, MO).

In Vitro *Cell Proliferation Assay*

The proliferation of 7ND-MSCs was compared with that of intact MSCs by using a colorimetric assay (TetraColor One; Seikagaku Co., Tokyo, Japan). MSCs (2000 cells per well) were seeded onto 96-well plates. After 72 hours of incubation, 10 μL of TetraColor One reagent was added to each well, and absorbance at 450 nm was measured 4 hours later. Percentage of proliferation was calculated as follows: (OD value of 7ND-MSC/OD value of intact MSCs) $\times$ 100.

Differentiation Assay

The multilineage potential of 7ND-MSC was confirmed as described elsewhere.[17] Briefly, intact MSCs, control (cont)-MSCs, and 7ND-MSCs were exposed to adipogenic formulas (R&D Systems, Minneapolis, MN) for 14 days or to osteogenic formulas (R&D Systems) for 21 days. Accumulation of intracellular lipid-rich vacuoles resulting from adipogenic differentiation was assessed by oil red O staining. Osteogenic differentiation was specifically evaluated by von Kossa staining to detect calcium deposition.

Purification and Quantification of 7ND

7ND was purified from the culture supernatants of 7ND-MSCs using the ANTI-FLAG M2 affinity gel system (Sigma-Aldrich). The 7ND content was measured using a specific human CCL2 enzyme-linked immunosorbent assay (eBioscience Inc., San Diego, CA).

Migration Assay

The inhibitory effect of purified 7ND on the CCL2-CCR2 axis was assessed using transwell assays.[18] Briefly, macrophage-like, CCR2-expressing RAW264.7 cells were serum starved in DMEM with 1% FBS overnight and were then seeded (5×10^5 cells in DMEM with 1% FBS per well) onto the upper chamber of a cell culture insert with a pore size of 8 μm (BD Biosciences, San Jose, CA). Recombinant mouse CCL2 (final concentration, 10 ng/mL; PeproTech, Rocky Hill, NJ) and various concentrations (final concentration, 0 to 1000 ng/mL) of purified 7ND were added to the lower chamber, to which cells were allowed to migrate for 4 hours. The membranes were fixed with 4% paraformaldehyde and were stained with Giemsa. For quantitative analysis, four fields were randomly selected, and migrated cells were counted under a light microscope.

Mouse Lung Injury Model

Lung injury was induced by endotracheal BLM injection as described elsewhere.[19] Briefly, mice were treated with 6 U/kg body weight of BLM diluted in sterile saline or the same volume of sterile saline only. Cont-MSC, 7ND-MSC (5×10^5 in 200 μL of PBS), or the same volume of PBS was injected into the tail vein 24 hours after BLM. Animals were sacrificed on days 2, 3, 7, and 14 (four to eight mice per subgroup on each day) or were untreated until day 28 (6 to 21 mice per subgroup) after BLM exposure. For histopathologic examination, lungs were fixed with 4% paraformaldehyde and were embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan). Otherwise, the minced left lungs were homogenized in 400 μL of PBS by using the Multi-Beads Shocker (Yasui Kikai, Osaka, Japan), and the minced right lungs were lysed in 400 μL of radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Calbiochem, La Jolla, CA). After removal of debris by centrifugation, the homogenates were used for collagen assay and the lysates were used for CCL2, IL-1β, and IL-6 measurements. Immunostain-

ing of 7ND-FLAG (day 2) was performed as reported previously.[16] Fibrosis was scored on sections of whole lungs (day 14) according the Ashcroft method.[20] Collagen content (days 14 and 28) in the left lung was evaluated by Sircol soluble collagen assay kit (Biocolor Ltd., Belfast, Ireland). Contents of CCL2, IL-1β, and IL-6 (day 14) in the right lung were assessed by Cytometric Bead Array flex sets (BD Biosciences).

RNA Preparation and RT-PCR Analysis

Total RNA was extracted from major organs of mice treated with cont- or 7ND-MSC using TRIzol (Invitrogen). cDNA was synthesized from total RNA using the SuperScript preamplification system (Invitrogen) and was used for RT–PCR. RNA quality was evaluated by parallel RT-PCR for glyceraldehyde-3-phosphate dehydrogenase. Primer sequences were as follows: human CCL2 sense: 5′-ATAGCAGCCACCTTCATTCC-3′, antisense: 5′-GTCTTCGGAGTTTGGGTTTG-3′; EGFP sense: 5′-AAGTTCATCTGCACCACCG-3′, antisense: 5′-TGCTCAGGTAGTGGTTGTCG-3′; and glyceraldehyde-3-phosphate dehydrogenase sense: 5′-GCCACCCAGAAGACTGTGGATGGC-3′, antisense: 5′-CATGTAGGCCATGAGGTCCACCAC-3′.

BAL Analysis

BAL samples were collected on days 3 and 7 after BLM as described elsewhere.[21] Differential counts of BAL cells were performed on 500 cells per cytospin slide.

Statistical Analysis

Analysis of variance was used to compare the means from two or more experimental groups, followed by Fisher's least significant difference post hoc tests. The survival benefit in mice treated with 7ND-MSC was analyzed

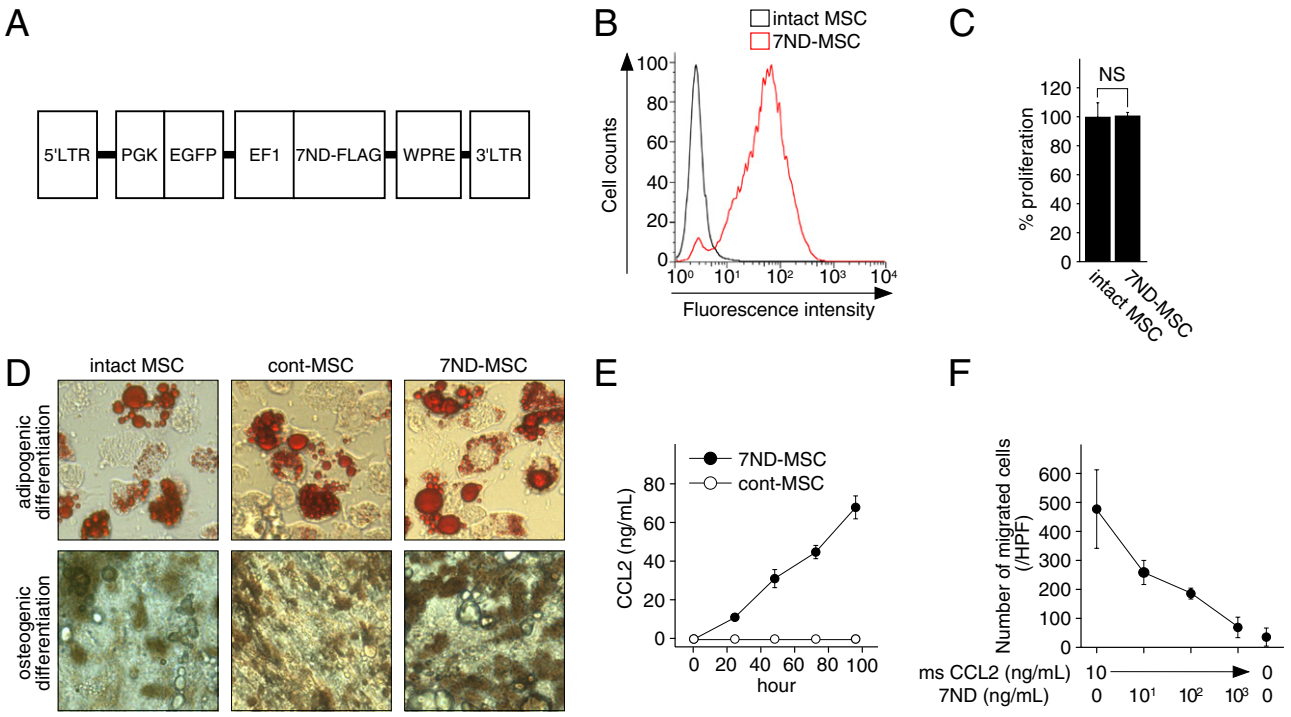

Figure 1. Characterization of MSCs stably transduced with a dominant-negative inhibitor of CCL2 (7ND). **A:** Structure of the pBGJR-EGFP-7ND-FLAG vector. A FLAG-tagged (3′ C terminus) deletion mutant of CCL2 (7ND) was recloned from the 7ND pCDNA3 expression vector into a lentiviral vector, which expresses EGFP (pBGJR-EGFP) under the control of the elongation factor-1 α promoter (EF1). LTR, long terminal repeat; PGK, phosphoglycerate kinase promoter; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element. **B:** Flow cytometric assessment of transduction efficiency. MSCs were incubated with the lentiviral vector shown in (**A**) in the presence of Polybrene (4 μg/mL), and transduction efficiency was determined by fluorescence-activated cell sorter analysis of EGFP expression (7ND-MSC). Nontransduced MSC (intact MSC) was used as a negative control. **C:** *In vitro* cell proliferation assay. The proliferation of 7ND-MSCs was compared with that of intact MSCs by using a colorimetric assay (TetraColor One). MSCs were seeded at a density of 2000 cells per well onto 96-well plates. After 72 hours of incubation, 10 μL of TetraColor One reagent was added to each well, and absorbance at 450 nm was measured 4 hours later. The percentage of proliferation was calculated as follows: (OD value of 7ND-MSC/OD value of intact MSCs) × 100. The results represent the mean ± SD of four replicates (representative experiment of three performed). **D:** Differentiation assay. To analyze the effects of lentiviral transduction of 7ND on the ability of MSCs to differentiate, we evaluated lipid deposition and calcium deposition, which are characteristic of adipogenic and osteogenic differentiation, respectively. Intact MSCs, cont-MSCs, and 7ND-MSCs were exposed to adipogenic formulas for 14 days or to osteogenic formulas for 21 days. Lipid depositions turn red after oil red O staining (**top panels**), and calcium depositions turn brown after von Kossa staining (**bottom panels**). Representative results of three independent experiments are shown. **E:** Quantification of 7ND secreted from 7ND-MSCs. Cont-MSCs or 7ND-MSCs were seeded at a density of 2000 cells per well in a 96-well plate. Culture supernatants were collected at the indicated time points. 7ND content was measured using a specific human CCL2 enzyme-linked immunosorbent assay. The results represent the mean ± SD of four replicates (representative experiment of three performed). **F:** Assessment of the inhibitory effect of 7ND on the CCL2-CCR2 axis. Mouse macrophage-like RAW264.7 cells expressing CCR2 were serum starved in DMEM containing 1% FBS overnight and were then seeded (5×10^5 cells in DMEM with 1% FBS per well) onto the upper chamber of a cell culture insert. Recombinant mouse CCL2 (final concentration, 10 ng/mL) and various concentrations of purified 7ND (final concentration, 0 to 1000 ng/mL) were added to the lower chamber, to which cells were allowed to migrate for 4 hours. The membranes were fixed with 4% paraformaldehyde and were stained with Giemsa. Four fields were randomly selected, and migrated cells were counted using a light microscope. The results reflect the mean ± SD of four independent determinations (representative experiment of three performed).

by the log-rank test. Statistical differences between groups were considered significant at $P < 0.05$.

Results

Stable Transformants Expressing 7ND Retain the Innate Properties of MSCs

The important domains in the 7ND-containing lentiviral vector are shown in Figure 1A. Characterization of MSCs transduced with 7ND-EGFP indicated that the transduction efficiency of 7ND was almost 100% (Figure 1B). EGFP fluorescence of the cells was consistently observed even after 2 months (data not shown). 7ND-MSCs can proliferate and differentiate into osteogenic and adipogenic lineages similar to intact MSCs (Figure 1, C and D). We analyzed the tissue distribution of MSCs after injection by RT-PCR to detect 7ND and EGFP in mice with 7ND-MSC and cont-MSCs, respectively. 7ND and EGFP were detectable strongly in the lung (prolonged for 14 days) and weakly in the liver and spleen on day 3 (not detectable on day 14) (data not shown). These results suggest that neither lentivirus nor 7ND perturbed the innate properties of MSCs.

Stable Transformants Secrete a Large Amount of 7ND that Is Biologically Active

7ND-MSCs secreted a large amount of 7ND (Figure 1E). Purified 7ND inhibited CCL2-induced migration of RAW264.7 cells dose dependently. Complete inhibition

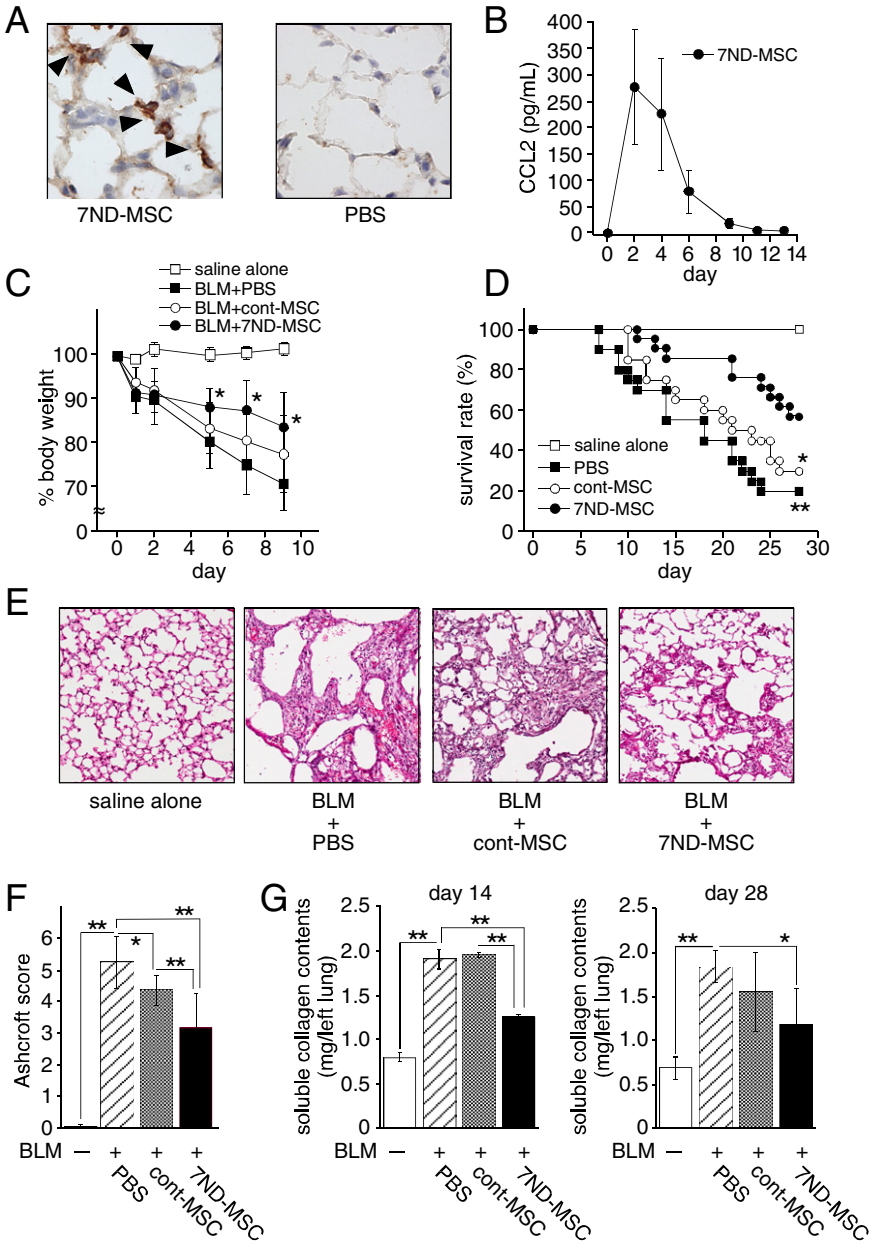

Figure 2. Efficacy of MSCs expressing 7ND in BLM-induced lung injury in mice. **A:** Detection of 7ND-MSCs in a mouse lung damaged by BLM. 7ND-MSCs (5×10^5) were injected intravenously 24 hours after BLM administration. The lungs were excised 2 days after BLM injection and were immunostained with an anti-FLAG antibody. Positive cells are indicated by **arrowheads** (**left panel**). Lungs from control animals that received PBS instead of 7ND-MSCs showed minimal background (**right panel**). Original magnification, ×40. Representative data of two independent experiments are shown. **B:** Plasma was collected periodically after the administration of 7ND-MSC to BLM-treated mice. The 7ND contents of the plasma were evaluated by a specific human CCL2 enzyme-linked immunosorbent assay. The results reflect the mean ± SD of five independent determinations (one determination from one mouse) and are representative of two independent experiments. **C:** Time course of body weight change after BLM. Mice (nine mice per subgroup) received cont-MSC, 7ND-MSC (5×10^5 in 200 μL of PBS), or the same volume of PBS the via tail vein 24 hours after challenge with BLM. Body weight change was monitored for up to 9 days after BLM injection. The results reflect the mean ± SD and are representative of three independent experiments. $^*P < 0.05$. **D:** Survival rates after BLM treatment. Mortalities were observed daily for up to 28 days after BLM-treated mice received saline alone (no BLM treatment, $n = 6$), BLM + PBS ($n = 20$), BLM + cont-MSC ($n = 20$), or BLM + 7ND-MSC ($n = 21$). The results are representative of three independent experiments. $P = 0.003$, BLM + PBS versus BLM + 7ND-MSC; $P = 0.045$, BLM + cont-MSC versus BLM + 7ND-MSC. $^*P < 0.05$, $^{**}P < 0.01$. **E:** Microscopic images of lungs after BLM treatment. Lungs removed on day 14 from mice that received saline alone (no BLM treatment), BLM + PBS, BLM + cont-MSC, or BLM + 7ND-MSC (eight mice per subgroup) were stained with H&E. Representative images of three independent experiments are shown. **F** and **G:** Assessment of lung fibrosis after BLM treatment. Fibrosis in lungs obtained on day 14 after BLM treatment was scored under a light microscope according to the Ashcroft method. The results reflect the mean ± SD of eight independent determinations (one determination from one mouse) (**F**). Lung fibrosis was also assessed as collagen accumulation (**G**). The left lobes were minced thoroughly and homogenized. After removing debris by centrifugation, the homogenates were used for collagen assay. The results reflect the mean ± SD of four independent determinations and are representative of two independent experiments. $^*P < 0.05$, $^{**}P < 0.01$.

of migration required 7ND at a 100-fold higher concentration than that of CCL2 (Figure 1F).

7ND-MSC Works as a Drug Delivery Tool

We identified many MSCs in BLM-treated lungs by immunostaining 24 hours after injection (Figure 2A). Controls showed minimal background. As described previously herein, 7ND expression was strongest in the lung. In another experiment, the serum level of 7ND was highest on day 2 after injection and was undetectable on day 11 (Figure 2B), suggesting that the number of MSCs diminished over time *in vivo*.

7ND-MSC Protects Mice from BLM-Induced Lung Injury and Fibrosis

We treated mice with 6 U/kg of BLM to mimic the human disease, although 1.25 to 4 U/kg of BLM is generally used.[22] This treatment resulted in severe weight loss, and approximately 45% of the mice died on day 14 (Figure 2, C and D). Weight loss was significantly lower in 7ND-MSC mice (Figure 2C). The survival rate on day 28 was significantly higher in 7ND-MSC mice (57.1%) than in BLM + PBS mice (20.0%) or cont-MSC mice (30.0%) (Figure 2D). BLM-treated mice developed subpleural inflammation with loss of normal alveolar, bronchi, and vasculature (Figure 2E). Administration of cont- and 7ND-MSCs reduced the extent of inflammation. However, significantly larger areas were undamaged in 7ND-MSC mice (Figure 2E), as confirmed by the Ashcroft method (Figure 2F). Accordingly, 7ND-MSC significantly reduced collagen content in the lung, whereas cont-MSC had a minimal effect (Figure 2G). We observed no evidence of MSC-induced toxicity.

7ND-MSC Efficiently Inhibits the Recruitment of Inflammatory Cells and the Production of Inflammatory Mediators

Next, we performed differential counts of BAL cells and quantified CCL2, IL-1β, and IL-6 in the lung. BLM but not saline significantly increased the accumulation of various kinds of inflammatory cells (Figure 3A). The numbers of total cells, macrophages, lymphocytes, eosinophils (except on day 3) and neutrophils (except on day 7) in BAL fluids were significantly lower in 7ND-MSC–treated mice (Figure 3A). Cont-MSC minimally inhibited neutrophil infiltration on day 3. 7ND-MSCs and cont-MSCs significantly decreased the lung content of IL-6 and IL-1β, but the 7ND-MSC–induced decrease was greater. 7ND-MSCs possessed a significantly higher ability to decrease CCL2 production than did cont-MSCs (Figure 3B).

Discussion

The evolution of viral vectors made it possible to deliver recombinant DNA molecules efficiently into host cells and let them be desirable for many applications. Especially, lentiviral vector constructs have proved to be very productive in terms of transduction owing to their ability to infect replicating and nonreplicating cells, including stem cells.[23] Characterization of 7ND-MSCs transduced by a lentiviral vector indicated that the transduction efficiency

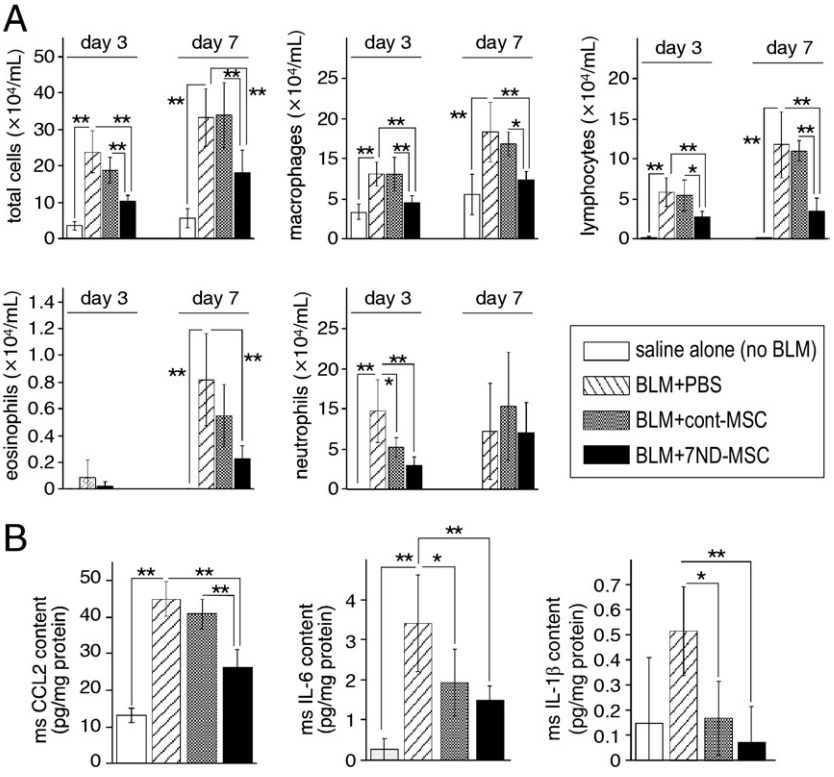

Figure 3. Mechanism of 7ND-MSC attenuation of BLM-induced lung injury. **A:** Cellular analysis of BAL fluid. BAL fluid specimens were obtained 3 and 7 days after BLM injection, and total and differential cell counts were performed (four mice per subgroup). The results reflect the mean ± SD and are representative of two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$. **B:** Assays for CCL2, IL-1β, and IL-6 in the lung. The right lobes of the lungs were removed on day 14 after BLM injection and were lysed in a radioimmunoprecipitation assay buffer. After removing debris by centrifugation, the lysates were used for CCL2, IL-1β, and IL-6 measurements. The results reflect the mean ± SD of five independent determinations (one determination from one mouse) and are representative of two independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$.

of 7ND was virtually 100% (Figure 1B) and that secretion of 7ND was very high (Figure 1E). We identified many MSCs in the BLM-treated lungs by immunostaining 24 hours after injection (Figure 2A). Because chemokines are locally retained on matrix heparan sulfate proteoglycans, thereby establishing a concentration gradient,[24] these results suggest that MSCs can be a useful 7ND vehicle for efficient inhibition of CCL2.

Persistence of MSCs *in vivo* may be tumorigenic[25]; however, we found that the 7ND serum level was highest on day 2 after injection and was undetectable on day 11 (Figure 2B), suggesting that the number of MSCs diminished over time *in vivo*. Therefore, an appropriate decrease in 7ND-MSCs over time could ensure clinical safety.

The numbers of total cells, macrophages, lymphocytes, eosinophils (except on day 3), and neutrophils (except on day 7) in BAL fluids were significantly lower in 7ND-MSC–treated mice (Figure 3A). Cont-MSC minimally inhibited neutrophil infiltration on day 3, similar to previous studies.[26,27] BLM-treated $CCL2^{-/-}$ mice and $CCR2^{-/-}$ mice showed a similar feature that infiltration of lymphocytes and macrophages, but not neutrophils, decreased in the lungs.[28,29] 7ND may attenuate lung fibrosis by inhibiting the recruitment of eosinophils that produced fibrogenic cytokines (Figure 3A).[30] Taken together, these results suggested that MSC and 7ND cooperatively inhibit accumulation of inflammatory cells. Subsequently, 7ND-MSCs significantly decreased the lung content of IL-6, CCL2, and IL-1β to a greater degree than did cont-MSCs (Figure 3B).

7ND gene transfer into muscles on day 7, but not on day 3, after BLM improved histopathologic findings.[31] In this study, administration of 7ND-MSCs 24 hours after BLM was effective. The highest concentration of 7ND was obtained on day 7 after 7ND gene transfer and on day 2 after 7ND-MSC administration. This discrepancy of effectiveness may result from different routes of 7ND administration, different local concentrations of 7ND in the lung, and different timing of 7ND production.

Mice were treated with 6 U/kg of BLM to mimic the human disease, with approximately 40% mortality, although 1.25 to 4 U/kg of BLM is generally used.[22] This treatment resulted in severe weight loss, and approximately 45% of the mice died on day 14 (Figure 2, C and D). The survival rate was significantly higher in 7ND-MSC mice (Figure 2D). Despite recent progress in medicine, mortality of patients with ARDS minimally decreased.[32,33] Thus, these data provide a rationale for testing 7ND-MSCs for patients with ARDS.

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