Supplementary material for the Online-Only Repository

SP-D regulates effector cell function and fibrotic lung remodeling in response to bleomycin injury

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Materials and Methods

Animals

Conditional or inducible (i) SP-D mice (FVB/N background) abbreviated as iSP-D mice, a triple transgenic mouse in which rat SP-D is expressed in response to doxycycline (Dox) [Clara cell secretory protein (CCSP)-reverse tetracycline transactivator transcription factor (rtTA+), $(tetO)_7$ -rat SP-D+, mouse SP-D(-/-)], and SP-D knockout (SP-$D^{-/-}$) mice (C57BL/6 background) were generated as previously described (1, 2). WT FVB/N, WT C57BL/6 mice, green fluorescent protein (GFP)-transgenic mice (C57BL/6 Background) were purchased from The Jackson Laboratory and bred in-house to account for environmental conditions. iSP-D mice were fed food containing Dox from birth. Dox was discontinued 1 week before bleomycin treatment for iSP-D mice in order to generate SP-D deficient mice. SP-D levels in BALF become lowest by day7 after Dox discontinuance (2, 3). Bone marrow (BM) transplanted mice were prepared as previously described with minor modifications (4). BM cells were collected from femurs of donor GFP-transgenic C57BL/6 mice by aspiration and flushing. Recipient C57BL/6 WT and SP-$D^{-/-}$ mice were exposed to two doses of 5 Gy given 3 hours apart using an X ray irradiator (attribute manufacturer), and then maintained on acidified water. After irradiation, 1×10^6 BM cells in 200 μl sterile PBS were injected retro-orbitally under anesthesia.

Isolation of recombinant SP-D

Recombinant SP-D was isolated from Chinese hamster ovary cells expressing a clone of the full-length rat SP-D gene, purified using maltose affinity chromatography, and

stored at 4°C in 5 mM Tris buffer, pH 7.8, containing 2 mM EDTA as described previously (5).

Bleomycin treatment

Mice were anesthetized with intraperitoneal injection with 150 mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 10 mg/kg xylazine (Lloyd Laboratories, Shenandoah, IA)， and mini-osmotic pumps (ALZET 2001, DURECT Corporation, CA) containing 200 μl saline alone (saline control) or containing 100 U/kg of bleomycin (Bedford Laboratories, Bedford, OH) were implanted subcutaneously (s.c.) in the left suprascapular lesion through an incision at the base of the neck. BLM was constantly infused from minipumps over 7 days as described in the manufacturer's.

Exogenous SP-D Administration In Vivo

Recombinant rat SP-D (2 μg in 50 μl saline) or 50 μl saline as control was administrated intratracheally into BLM-treated SP-D$^{-/-}$ mouse with oropharyngeal aspiration method as previously described (6) twice a week from days 0 to 28 to determine whether exogenous SP-D could reduce BLM-induced lung fibrosis on day28. Briefly, the anesthetized mice were suspended by the upper incisors with a thin wire on a 60° incline board. The tongue was gently extended and saline with or without SP-D was pipette into the mouth. Brief occlusion of the nose forced the animal to inhale through the mouth, thereby aspirating the solution into the respiratory tract in one or two breaths. Animals were subsequently removed from the board and observed closely until fully recovered from anesthesia.

Bronchoalveolar lavage

For bronchoalveolar lavage (BAL), mice were anesthetized with pentobarbital and the lungs and heart were surgically exposed. The trachea was canulated and the lungs were lavaged 6 times with 1 ml of 0.1 mM EDTA/PBS. Total BAL cells were collected by centrifugation, and the live cells were counted using a hemocytometer. Cytospin preparations of BAL cells were stained with Hemacolor (EMD Chemicals, Gibbstown, NJ) and differential cell counting was performed. The supernatant of the first 1 ml of recovered BAL fluid was saved and stored at -80℃ for cytokine measurement and Western blot analysis. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Duke University Medical Center.

Collagen assay

Total lung collagen was determined using the Sircol Collagen Assay kit (Biocolor Ltd., Belfast, United Kingdom) according to the manufacturer's instructions (7). Briefly, left lungs were harvested on day 35 after implantation of the minipumps containing BLM and homogenized in 0.5 M acetic acid (50 volumes to wet lung weight) containing about 1 mg pepsin/10 mg tissue residue. Each sample was incubated for 24 hours at room temperature with stirring. After centrifugation, 100 μl of each supernatant was assayed. One milliliter of Sircol dye reagent, which binds to collagen, was added to each sample, and then mixed for 30 minutes. After centrifugation, the pellets were suspended in 1 ml of the alkali reagent included in the kit and read at 540nm in a spectrophotometer.

Collagen standard solutions were utilized to construct a standard curve. Collagens contain about 14% hydroxyproline by weight, and collagen contents obtained with this method correlate well with hydroxyproline content according to the manufacturer's data.

Histopathology

The right lungs were fixed in 10% buffered Formalin and embedded in paraffin. Sections (3 to 4 μm) were stained with hematoxylin and eosin, and then examined by light microscopy. To evaluate further the fibrotic changes (collagen and elastin), Masson's-trichrome staining was performed. The severity of the fibrotic changes in each lung section was assessed as a mean score of severity from observed microscopic fields. Fifteen fields within each lung section were evaluated at a magnification of x100. Each field was assessed individually for the severity of fibrotic changes and given a score of 0 (normal) to 8 (total fibrosis). The mean score of all evaluated fields was considered the fibrotic score.

Immunohistochemistry

On day 7, 21 and 28 after BLM treatment, the harvested lungs were fixed in 10% buffered formalin and placed into OCT compound (SAKURA Finetek, Torrance, CA) and snap frozen for immunohistochemical analysis. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method was performed after the protocol of TACS 2 TdT-Fluor In Situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD). Briefly, the slides were washed with PBS and permeabilized with Cytonin. DNA strand breaks were then end-labeled with terminal transferase, and the labeled DNA

was visualized using Streptavidin-fluorescein isothiocyanate (FITC). Ten random fields per sample were counted for TUNEL-positive cells by a confocal laser scanning microscope (Zeiss LSM 710, Zeiss, Germany, magnification ×20). The number of 4',6-diamidino-2-phenylindole (DAPI)-positive (nucleated) cells was also counted, and TUNEL-positive index was calculated by dividing the TUNEL-positive by DAPI-positive cell numbers. For immunohistochemistry for transforming growth factor-β1 (TGF-β1), platelet-derived growth factor (PDGF)-A, F4/80, GFP, fibroblast-specific protein-1(FSP-1)/S100A4 and SP-C, the sections were blocked for 1 hour at room temperature in PBS contained 5.0% bovine serum albumin. The sections were stained with primary antibodies at 4°C overnight and subsequently stained with fluorescence-conjugated secondary antibodies and DAPI at room temperature for 1 hour. Fluorescence images of sections excited at wavelengths of 405 nm, 488 nm and 594 nm were captured with a conforcal laser scanning microscope (magnification ×20) (attribute manufacturer – Leica, Zeiss etc).

Fibrocyte isolation

Murine fibrocytes were isolated from the lungs according to previously published methods (8). Briefly, the lungs were harvested from saline or BLM-treated mice and minced with scissors. The minced lungs were digested by 1 mg/ml of collagenase A (Roche) and 100 μg/ml of DNase I (Worthington/Sigma), and the cells were collected by centrifugation. The harvested cells were cultured in DMEM supplemented with 20% fetal bovine serum (FBS) (GIBCO BRL, Rockville, MD). Lung mesenchymal cells were grown from lung digests of mice for 7-10 days on fibronectin-coated 25 cm^2 flasks. The

adherent cells were harvested by using 0.05% trypsin. We used immunomagnetic selection to isolate fibrocytes (CD45-positive mesenchymal cells). The trypsinized cells were stained with anti-CD45 Abs coupled to magnetic beads (Miltenyi Biotech, Auburn, CA). Labeled cells were then sorted by binding the cell population to positive selection columns using AutoMacs (Miltenyi Biotech).

Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assays (ELISA) for mouse Tumor necrosis factor-α (TNF-α), interleukin (IL)-1β (BD Biosciences, Franklin Lakes, NJ), TGF-β1, PDGF-AA, PDGF-BB (R&D Systems, Minneapolis, MN) were performed according to manufacturer's guidance with BAL samples from indicated time points. Latent TGF-β1 was activated to immunoreactive TGF-β1 with 1N HCl in BAL fluids before performing ELISA for TGF-β1.

FACS analysis

The lungs were harvested from saline or BLM-treated mice on day 0, 14 or 21 and minced with razor blades. The minced lungs were treated with collagenase A and DNase I to produce single cell suspensions that were initially stained with FITC labeled anti-mouse CD45 Ab and PE labeled anti-mouse CXCR4 Ab. Subsequently, the cells were fixed in 10% neutral buffered formalin, permeabilized by 0.3% saponin and then stained with biotin-conjugated anti-mouse collagen I Ab (Rockland, Gilbertsville, PA), followed by Streptavidin-Texas Red. The stained cells were analyzed by FACS using a BD LSRII (San Diego, CA) for acquisition and FloJo software (Treestar, Inc. OR) for

analysis.

Quantitative real-time PCR analysis

Total RNA was extracted from murine fibrocytes isolated from the lungs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reversed transcribed into cDNA according to the manufacturer's instructions. Quantitative real-time PCR amplification was performed using .i-Cycler iQ Detection System (Bio-Rad, Hercules, CA). The standard curve method was used to quantify the expression of the target genes and cyclophilin in each sample. The specificity of the PCR products was confirmed by melting curve analysis. The normalized results were expressed as the ratio of the target gene to cyclophilin.

Western blot analysis

Equal volumes of BAL fluid samples (day 0, 7, 14, 21, 35) from saline or BLM-treated mice were resolved under reducing conditions by 10% SDS-PAGE, transferred onto nitrocellulose membrane, which was blocked with 3% bovine serum albumin. The membrane was probed with 1:2000 of rabbit anti-mouse SP-D or 1:2000 of rabbit anti-sheep SP-A (cross reacts to mouse SP-A), followed by 1:4000 of goat anti-rabbit Alexa488 secondary antibody. The image was acquired by Typhoon imager (GE Healthcare, Buckinghamshire, UK) at 800 lines of resolution.

Statistical Analysis

Comparisons among multiple groups were analyzed using the one-way analysis of variance with Newman-Keuls *post hoc* correction (GraphPad Prism, version 5.0; GraphPad Software, Inc., San Diego, CA). Differences were considered statistically significant if p values were less than 0.05.

References

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FIGURE LEGENDS

Figure E1

Recruited macrophages into the lungs are dominant in bleomycin-treated FVB/N WT mice compared to C57BL/6 mice. BAL fluid was collected from FVB/N WT mice or C57BL/6 WT mice on Day 0, 7, 14 or 28 after BLM treatment. (A) Total cell and (B-D) differential cell counts were performed. PMNs = polymorphonuclear neutrophils. Data are presented as mean ± SEM. n=4 in each group.

Figure E2

Fibrocytes were increased in the lungs of BLM-treated SP-D$^{-/-}$ mice. (A) Col-I+ CXCR4+ CD45+ fibrocytes in lung digests from saline or BLM-treated C57BL/6 WT

mice or BLM-treated SP-D$^{-/-}$ mice on day 14 were examined by FACS. (B) The percent fibrocytes relative to total cells of lung digests ± SEM n=3-4 in each group.

Figure E3

SP-D levels in BAL fluid after SP-D administration in SP-D$^{-/-}$ mouse. BAL fluid was collected from SP-D$^{-/-}$ mice at 1hr, 24hrs or 48hrs after 2 μg of rat SP-D administration on day 7 after saline or BLM treatment, untreated SP-D$^{-/-}$ mice at 5 min after the administration or untreated C57BL/6 WT mice. (A) Equal amount of BAL fluid from each mouse was resolved by SDS-PAGE, and SP-D was detected by Western blot analysis. (B) The average intensities of SP-D bands relative to that of WT mouse in each group are shown. Data are presented as mean ± SEM. n = 3

Figure E4

SP-D expression (Dox on) during fibrotic phase attenuates BLM-induced lung fibrosis. (A) Typical photomicrographs of H&E staining of the lungs from iSP-D mice off Dox (SP-D off), on Dox continuously (SP-D on), on Dox during early phase (birth-day14) or on Dox during fibrotic phase (day14-35) after BLM treatment. Magnification ×10. (B) Evaluation of fibrotic change in the lung using numeric fibrotic score.
Data are presented as mean ± SEM of all fields examined in each group of 4-5 mice.

Figure E1

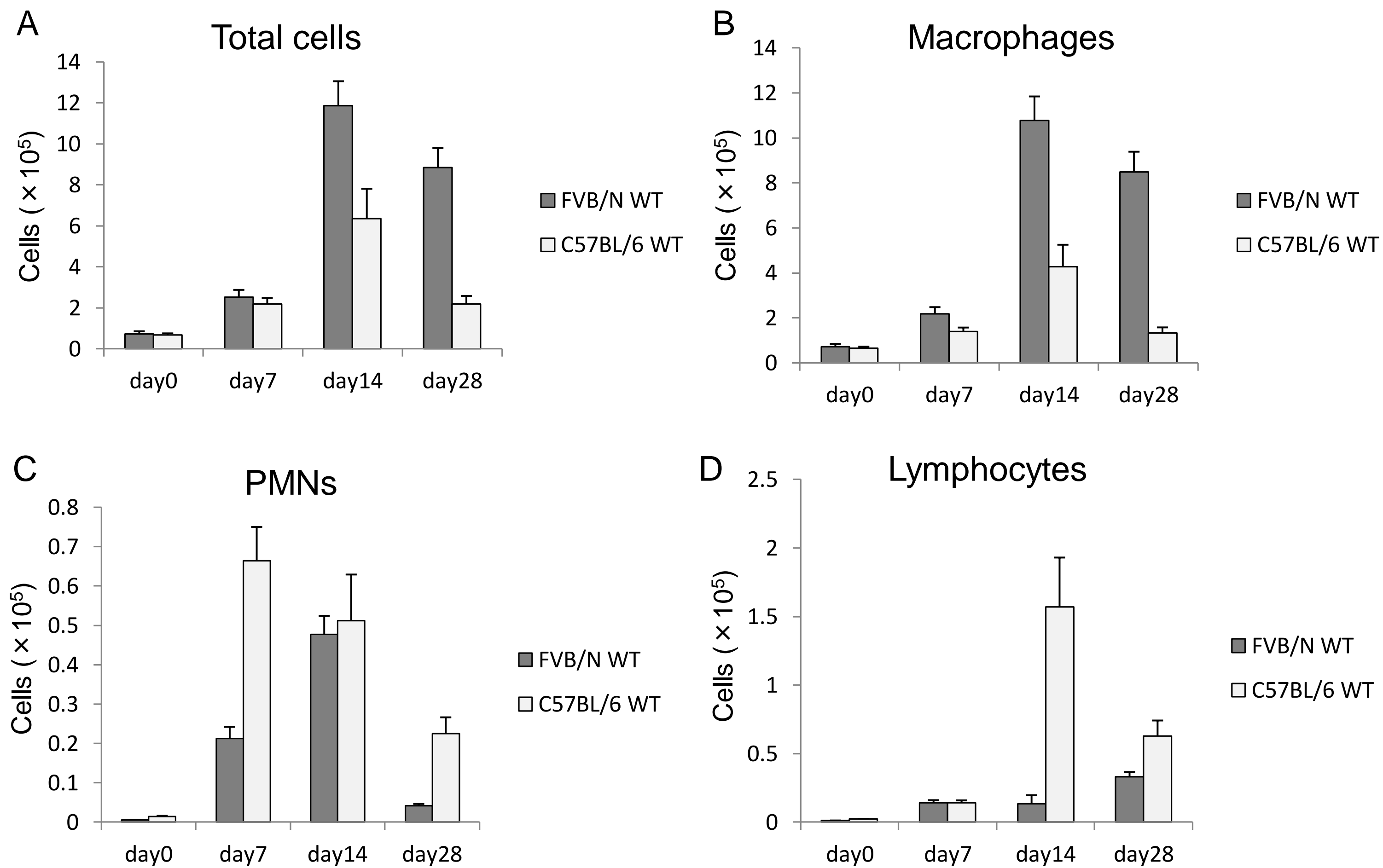
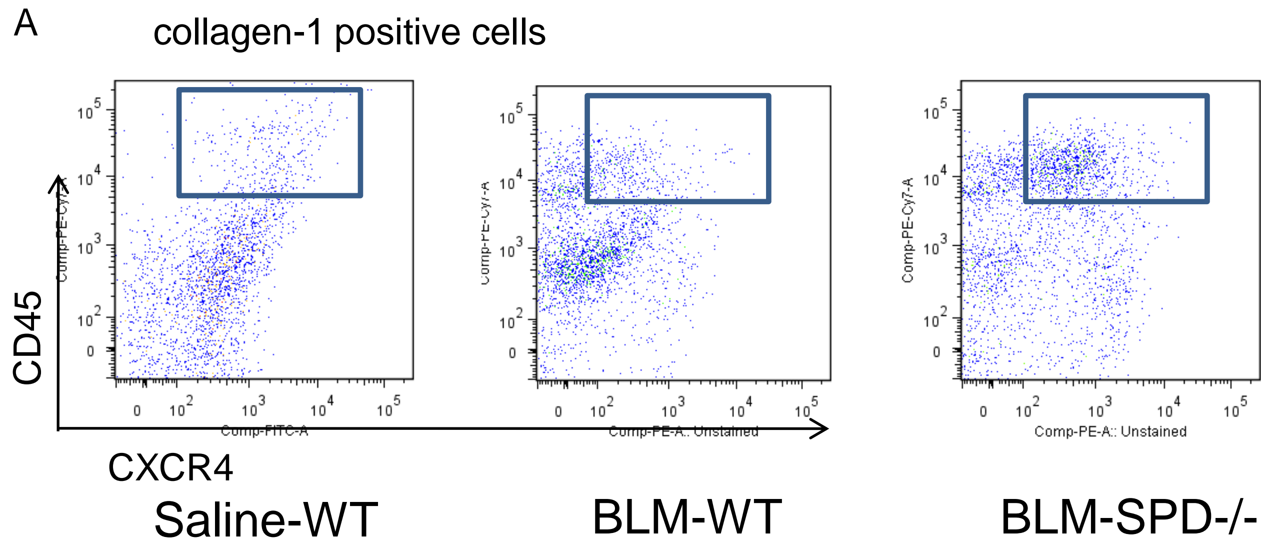


Figure E2



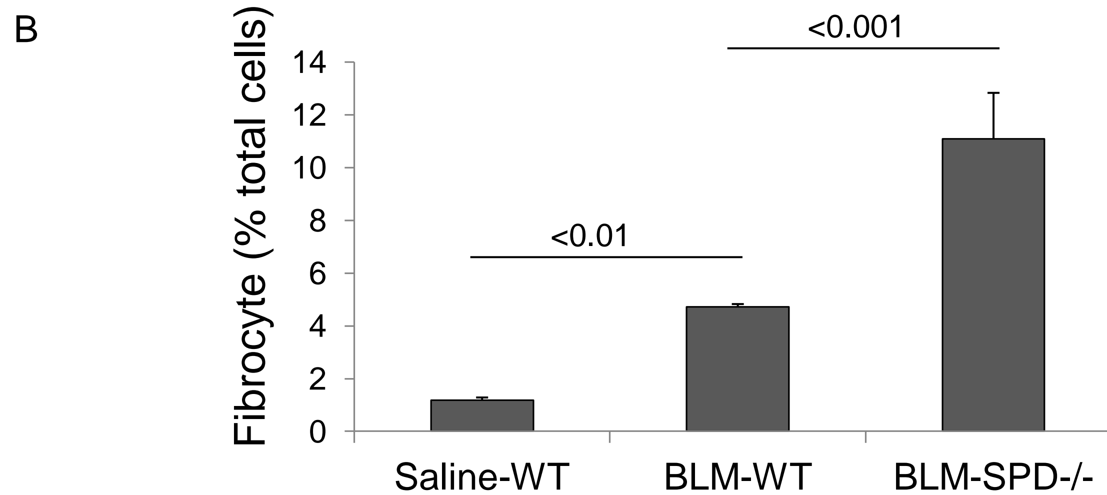
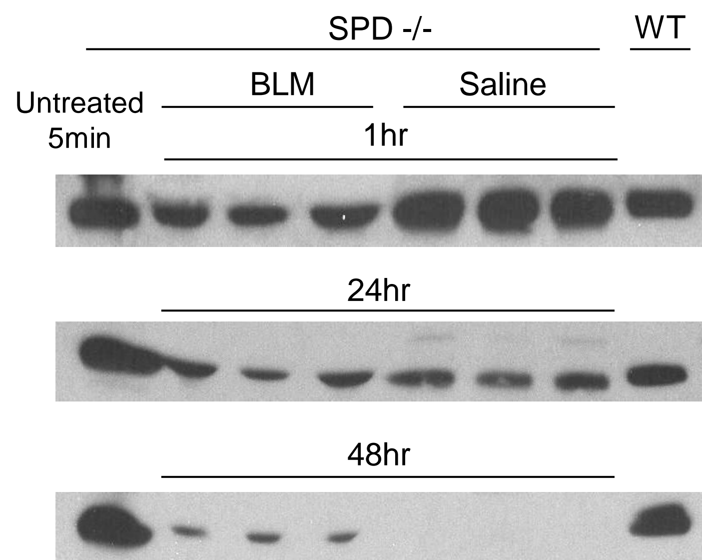


Figure E3



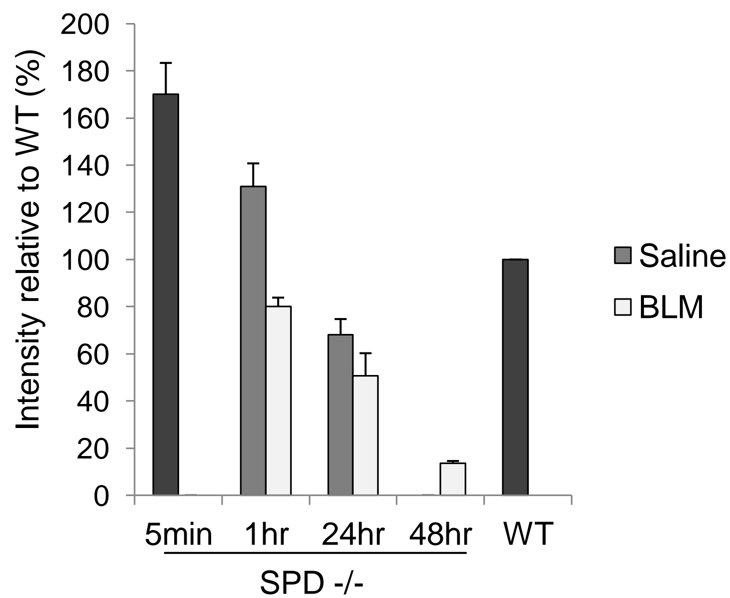


Figure E4