ONLINE DATA SUPPLEMENT

Expression of Stromal Cell-Derived Factor-1 by Mesenchymal Stromal Cells Impacts Neutrophil Function During Sepsis

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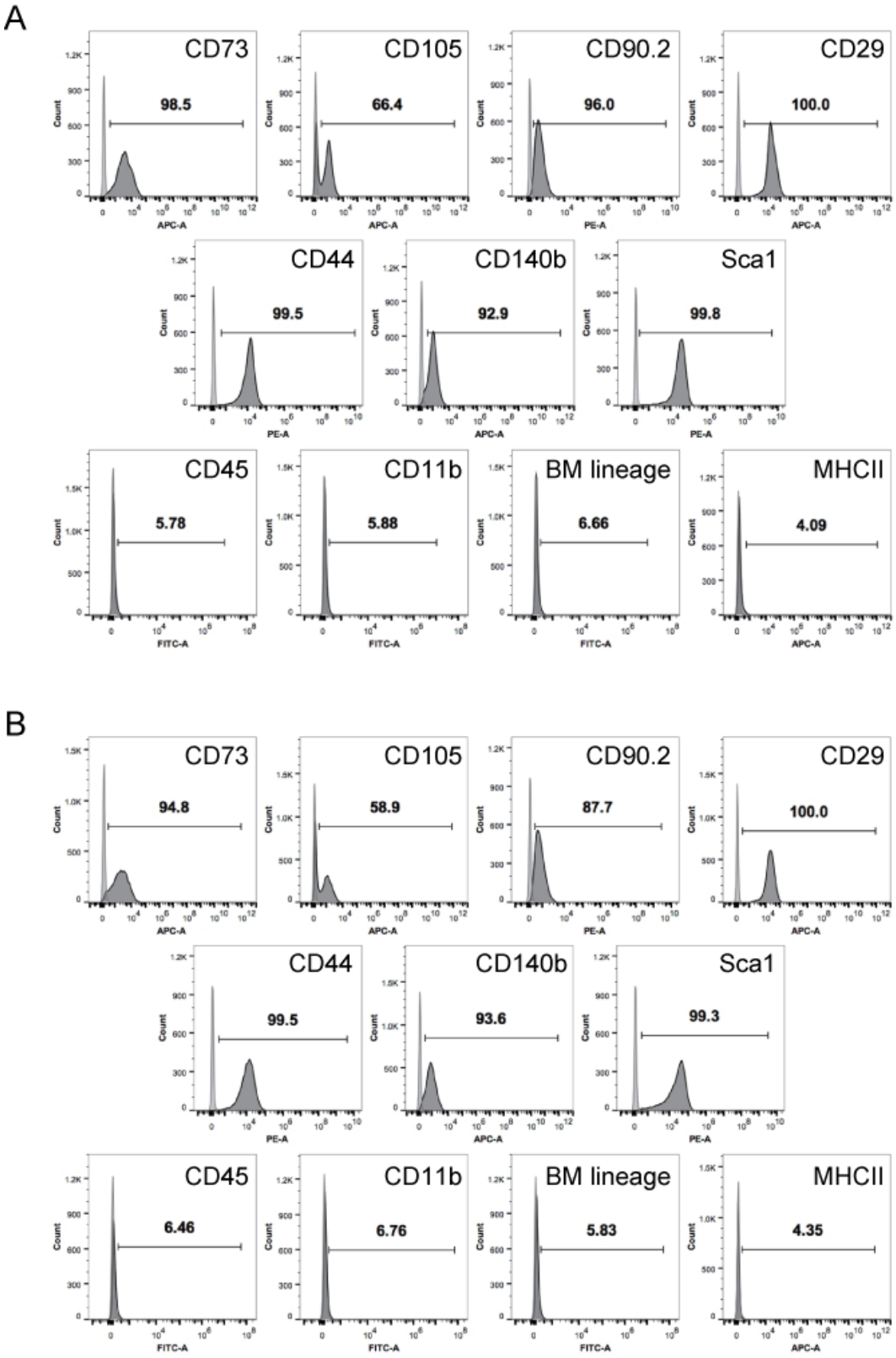
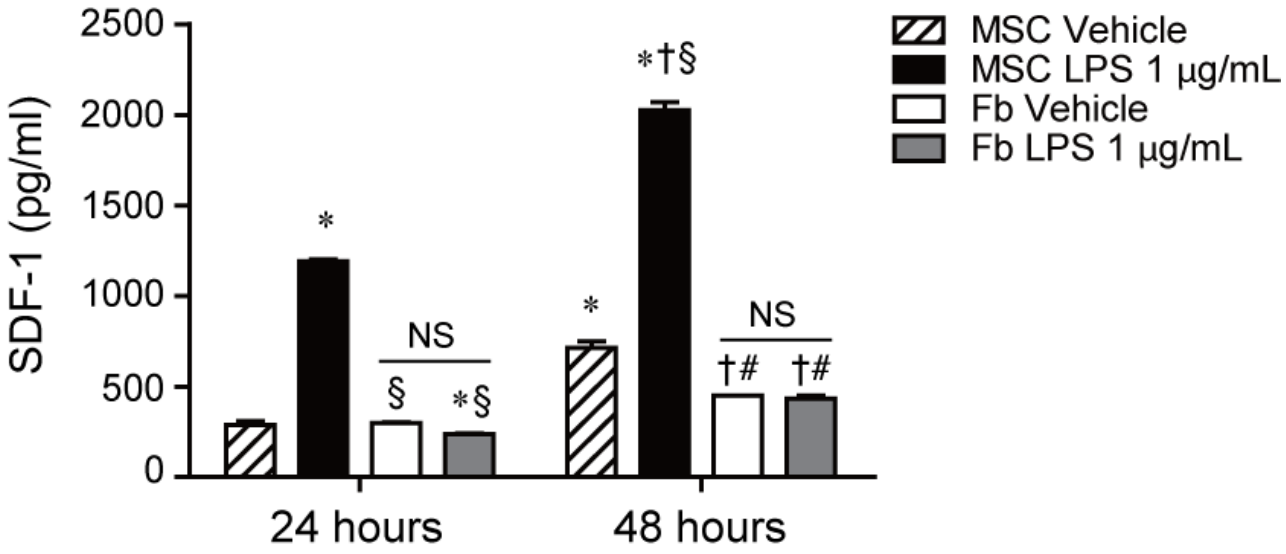
Supplemental Figure 1



Supplemental Figure 2



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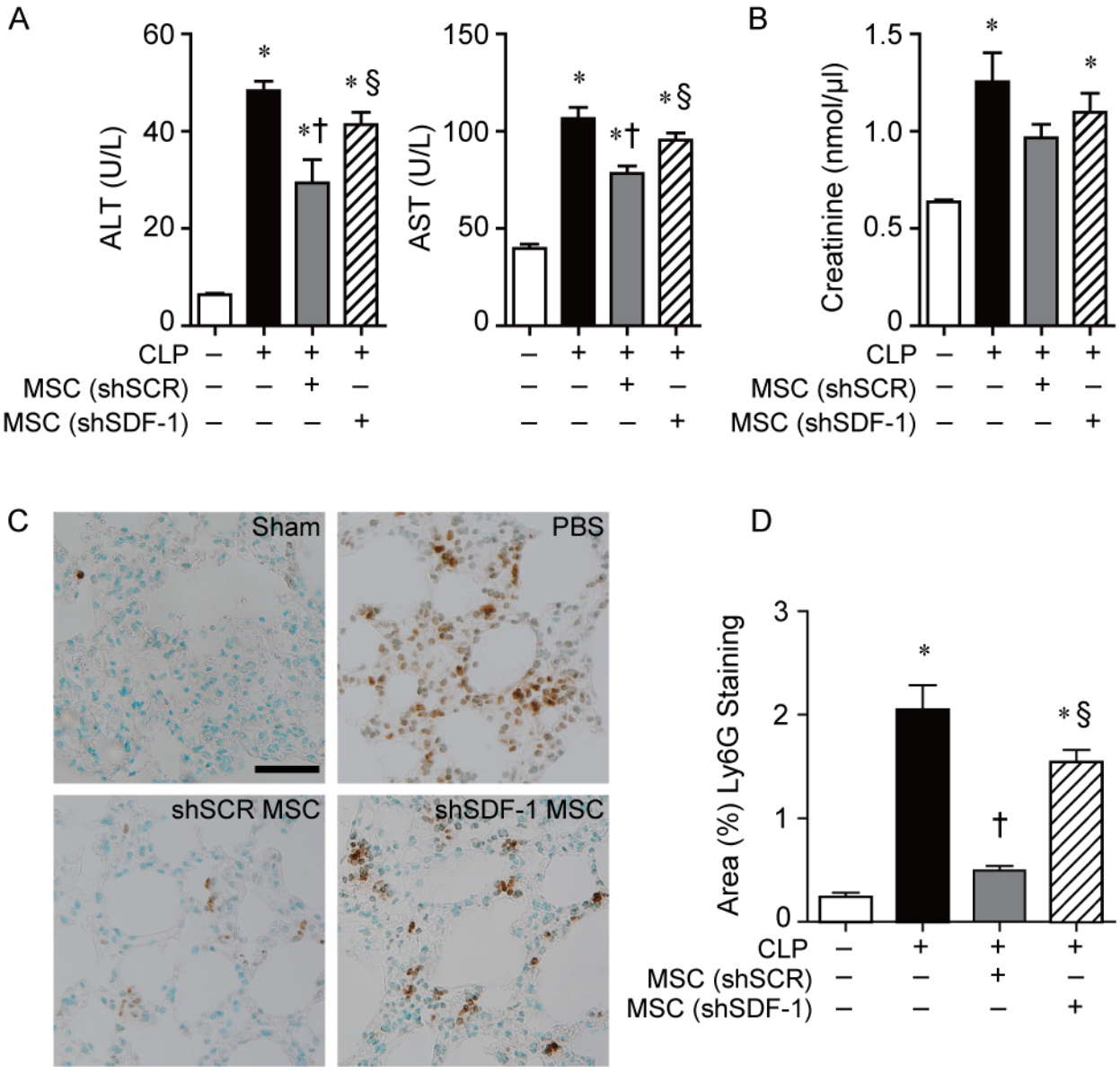

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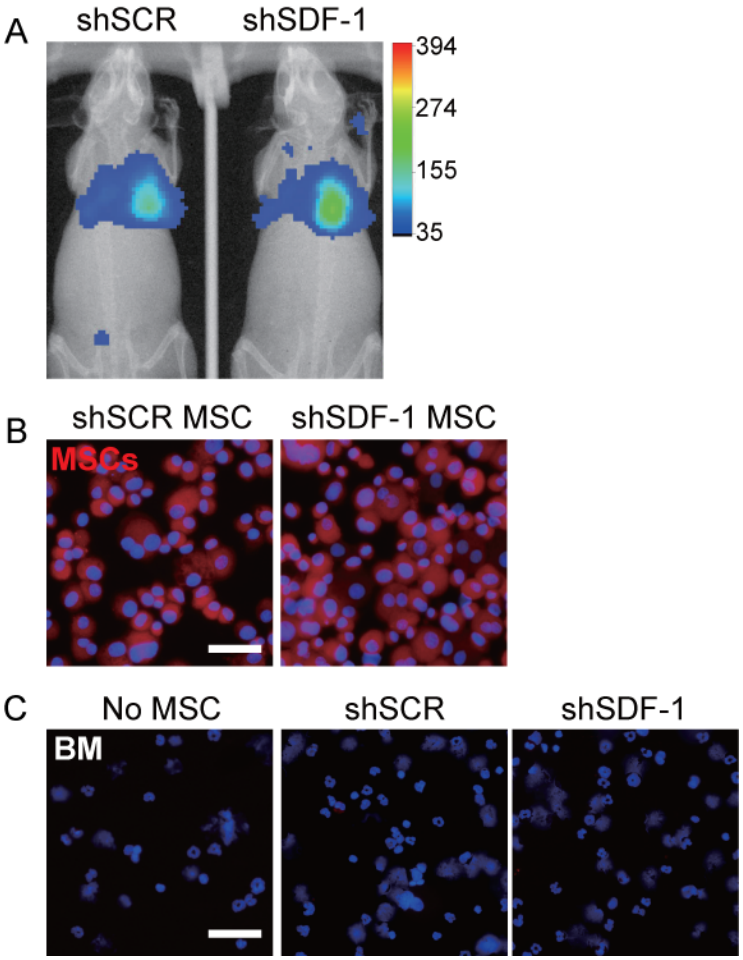

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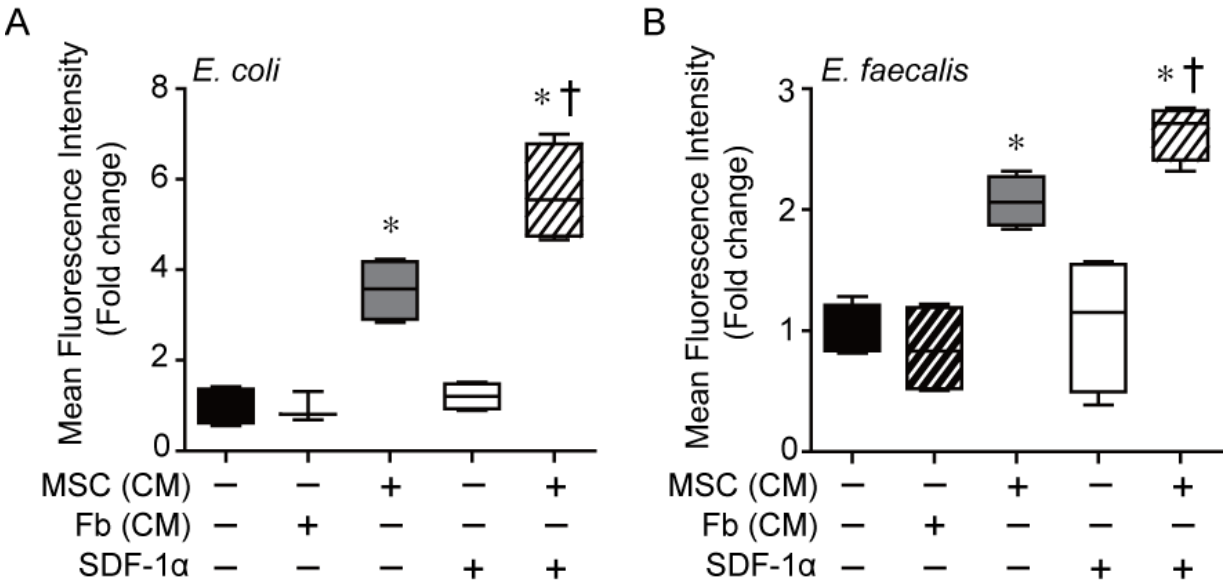

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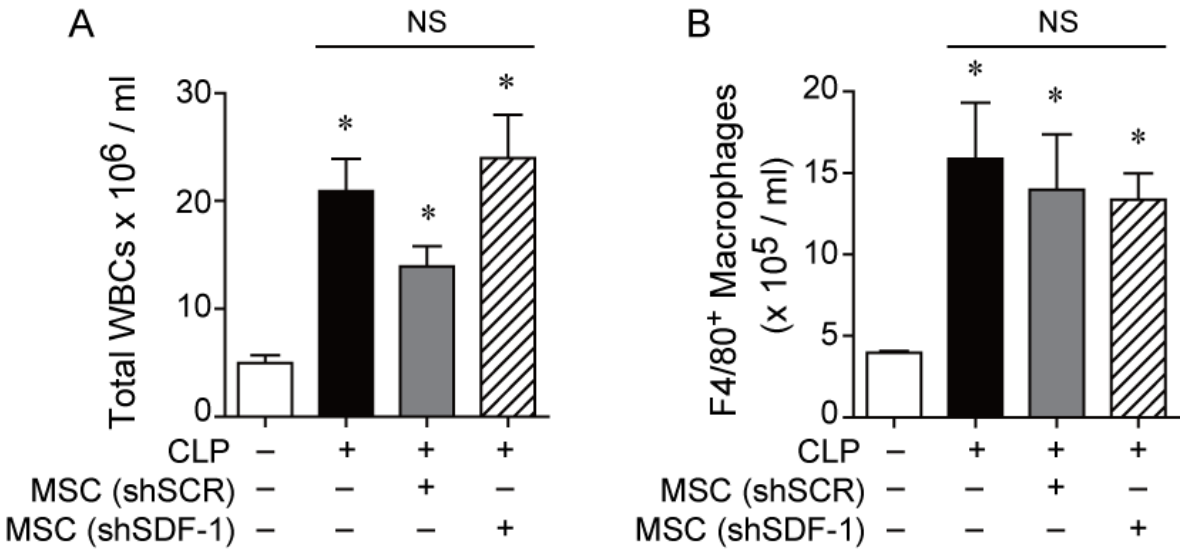

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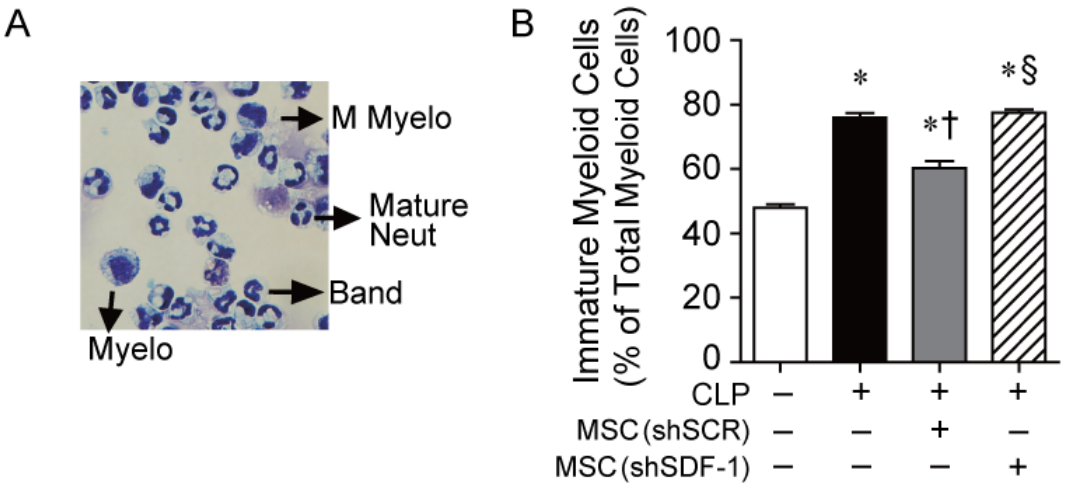
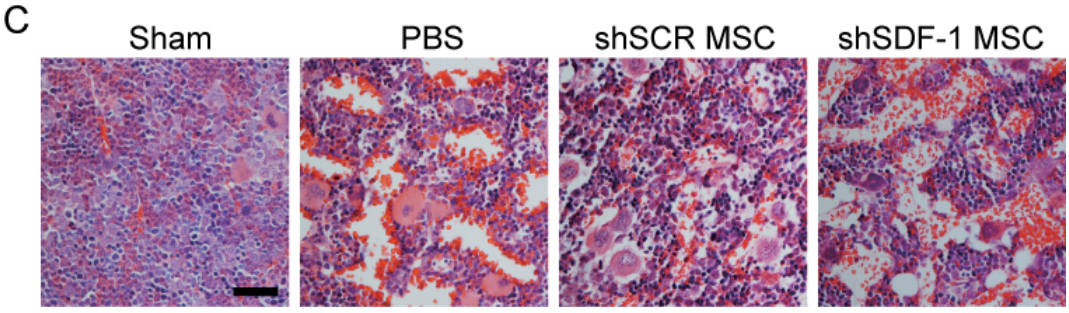
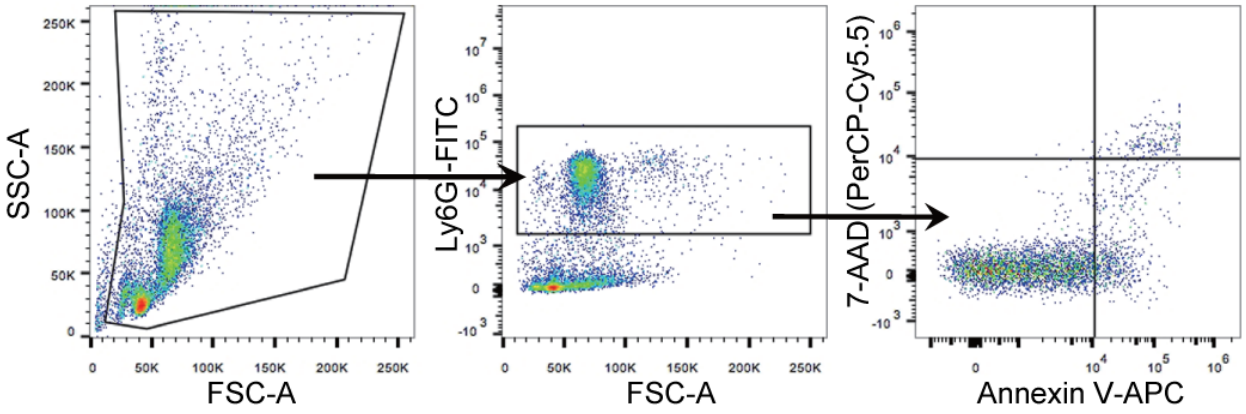


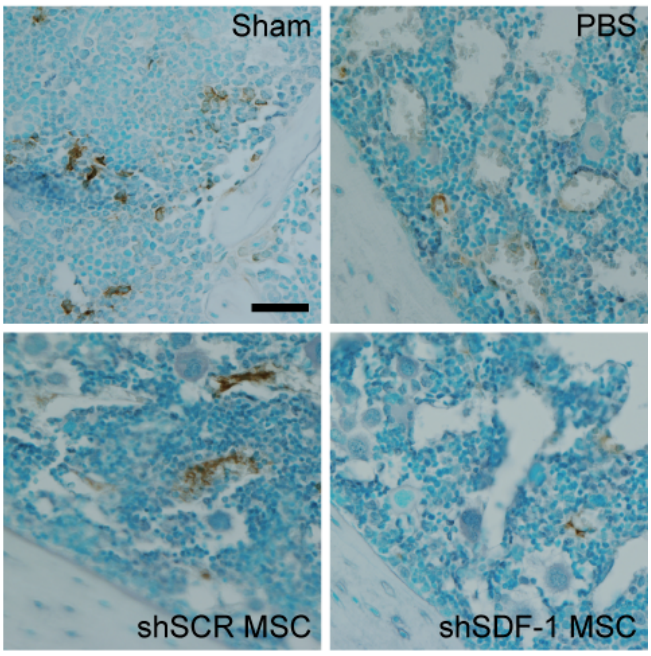


Supplemental Figure 8



Supplemental Figure 9

A



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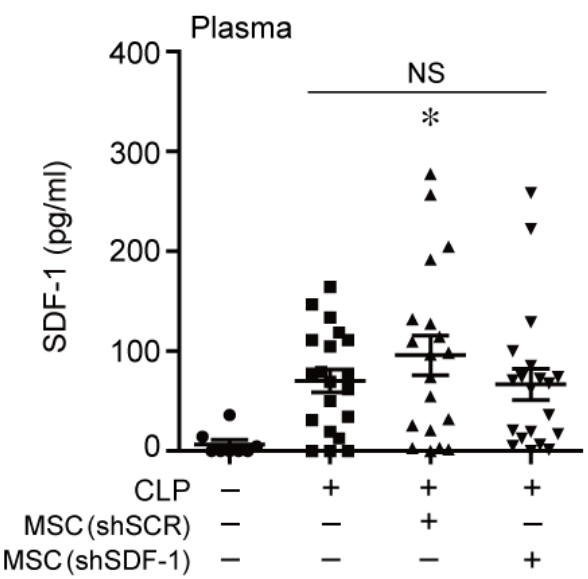

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Flow cytometry characterization of MSCs. Cells were harvested from the compact bones (femurs and tibias) of BALB/c mice. After expansion and depletion of the hematopoietic lineage cells, the putative MSCs were infected with either a scrambled construct (shSCR, **A**) or silenced for SDF-1 (shSDF-1, **B**). Cells were assessed for mesenchymal markers (CD73, CD105, CD90.2, CD29, CD44, CD140b), hematopoietic origin markers (CD45, CD11b, bone marrow lineage markers), Sca1, and MHC II. Representative histograms are shown for shSCR MSCs (**A**) and shSDF-1 MSCs (**B**), with staining for isotype control antibodies (light gray) and target antibodies (dark gray).

Supplemental Figure 2. Wild-type MSCs, but not fibroblasts, exposed to lipopolysaccharide (LPS) led to an increase in SDF-1 levels in the culture medium. Wild-type MSCs and lung fibroblasts were exposed to vehicle or LPS (1 μg/ml) for 24 and 48 hours. The cell culture supernatants were then collected and SDF-1 assessed by ELISA, in pg/ml concentrations. MSCs exposed to vehicle (striped bar) or LPS (black bar), and Fibroblasts exposed to vehicle (white bar) or LPS (gray bar) are depicted at 24 and 48 hours after stimulation in four independent experiments (n=4). Data are represented as mean±SEM. $P<0.0001$, with significant comparisons * versus MSC vehicle 24 hours, † versus MSC vehicle 48 hours, § versus MSC LPS 24 hours, and # versus MSC LPS 48 hours.

Supplemental Figure 3. Mice receiving shSDF-1 MSCs after the onset of CLP-induced sepsis have more organ injury (liver and kidney) and more neutrophils in the lungs

compared with mice treated with shSCR MSCs. Mice underwent Sham or CLP-surgery. In the septic mice, they received either PBS, shSCR MSCs, or shSDF-1 MSCs 2 hours after CLP surgery. **A**) Plasma alanine aminotransferase (ALT, left panel) and aspartate aminotransferase (AST, right panel), and **B**) plasma creatinine were assessed 24 hours after Sham or CLP surgery. Sham, white bar, n=6; CLP+PBS (– MSCs), black bar, n=6; CLP+shSCR MSCs, gray bar, n=6; CLP+shSDF-1 MSCs, striped bar, n=6. $P<0.0001$ (ALT and AST) and $P=0.0008$ (creatinine), with significant comparisons * versus Sham, † versus CLP+PBS (– MSCs), § versus CLP+shSCR MSCs. **C**) 24-hours after Sham or CLP surgery, the lungs were harvested and immunostaining was performed with Ly6G antibody (brown) to assess neutrophils. Left upper panel, Sham; right upper panel, CLP+PBS; left lower panel CLP+shSCR MSCs; and right lower panel CLP+shSDF-1 MSCs. The scale bar (Sham) represents 25 μm. **D**) The area of Ly6G positively stained cells was calculated per 20X objective of lung tissue, and random fields were assessed per tissue section of Sham (white bar, n=14), CLP+PBS (– cells, black bar, n=29), CLP+ shSCR MSCs (gray bar, n=31) and shSDF-1 MSCs (striped bar, n=41). Data are represented as mean±SEM. $P<0.0001$, with significant comparisons * versus Sham, † versus CLP+PBS (– MSCs), § versus CLP+shSCR MSCs.

Supplemental Figure 4. MSCs predominantly remain in the thoracic cavity, and do not reach the bone marrow, during CLP-induced sepsis. **A**) MSCs (shSCR and shSDF-1) were tracked by bioluminescence after intravenous administration 2 hours following CLP, and assessed at 36 hours, showing that the predominance of cells remain in the thoracic cavity in both groups. **B**) shSCR and shSDF-1 MSCs were stained with the red fluorescent

dye PKH26 (scale bar represents 25 μm), and **C**) injected intravenously into mice 2 hours following CLP. At 36 hours after CLP surgery, mice receiving no MSCs, shSCR MSCs, or shSDF-1 MSCs were sacrificed and bone marrow cells were collected from femurs and tibias, and cytospins of the cells were performed followed by staining with DAPI (blue). The scale bar represents 25 μm.

Supplemental Figure 5. SDF-1α mediates MSC-enhanced neutrophil phagocytosis. Neutrophils were incubated with GFP-labeled *E. coli* (**A**) or *E. faecalis* (**B**) in the presence of no stimulus (–, black bar), conditioned media (CM) from fibroblasts (Fb, black bar with white stripes), CM from MSCs (gray bar), SDF-1α alone (white bar), or SDF-1α plus CM from MSCs (white bar with black stripes). n=4 per group from independent experiments. $P<0.0001$ with significant comparisons * versus neutrophils alone or neutrophils+Fb CM or neutrophils+SDF-1α; † versus neutrophils+MSC CM.

Supplemental Figure 6. Modulation of the inflammatory response by MSC-derived SDF-1; no significant difference in total white blood cells (WBCs) or macrophages from the peritoneum of mice after CLP-induced sepsis. **A**) Total WBC count was performed from the peritoneal fluid of Sham (white bar, n=5) or septic BALB/c mice 24 hours after surgery, and were randomly separated into 3 groups: PBS control (black bar, n=5), shSCR MSCs (gray bar, n=5), and shSDF-1 MSCs (striped bar, n=5). Mice subjected to CLP received PBS or cells (5×10^5) via tail vein injection 2 hours after CLP. $P=0.0019$, with significant comparisons * versus Sham. NS=not significant. **B**) The peritoneal fluid

was assessed by flow cytometry for F4/80$^+$ macrophages from the groups described in (**A**). *P*=0.0154. * versus Sham. NS=not significant.

Supplemental Figure 7. Mice receiving shSDF-1 MSCs after the onset of CLP-induced sepsis have more immature cells in the peritoneum, and more hypocellular bone marrow, than mice treated with shSCR MSCs. **A**) Cytospins of peritoneal fluid were performed on mice 24 hours after CLP-induced sepsis. Hema 3 staining was performed. The image denotes mature neutrophils (Mature Neut), banded neutrophils (Band), myelocytes (Myelo) and metamyelocytes (M Myelo). **B**) Mice were subjected to Sham or CLP surgery, and 2 hours after CLP the mice received PBS or cells (5 X 10^5) via tail vein injection. Sham (white bar, n=9), PBS (black bar, n=14), shSCR MSCs (gray bar, n=15), and shSDF-1 MSCs (striped bar, n=16). Cytospins were performed and immature neutrophils were assessed as a percentage of total myeloid cells. Data are represented as mean±SEM. *P*<0.0001, with significant comparisons * versus Sham, † versus CLP+PBS (– MSCs), § versus CLP+shSCR MSCs. **C**) Histologic assessment of bone marrow from mice exposed to Sham or CLP-induced sepsis was assessed at 24 hours after surgery. H&E staining was performed on sections of femurs from Sham mice, or mice that underwent CLP and received PBS, shSCR MSCs, or shSDF-1 MSCs. The scale bar (Sham) represents 25 μm.

Supplemental Figure 8. Gating strategy for apoptosis of Ly6G$^+$ neutrophils in the bone marrow. Bone marrow was harvested, and the cells were stained with Ly6G-FITC antibody, Annexin V-APC antibody, and 7-AAD dye (PerCP-Cy5.5). For flow cytometry,

the cells were first gated for forward and side-scatter (left panel), and then identified as staining positive for Ly6G (middle panel). Finally the cells were assessed for staining of Annexin V and 7-AAD (right panel).

Supplemental Figure 9. SDF-1 is more highly expressed in the bone marrow of mice treated with shSCR MSCs after the onset of CLP-induced sepsis, compared with mice receiving shSDF-1 MSCs. **A**) Bones (femurs) were harvested at 24 hours after CLP. Immunostaining was performed with SDF-1 antibody (brown) on sections of the femurs. Left upper panel, Sham; right upper panel CLP+PBS; left lower panel CLP+shSCR MSCs; and right lower panel CLP+shSDF-1 MSCs. The scale bar (Sham) represents 25 μm. **B**) SDF-1 protein levels were also assessed in plasma at 24 hours after CLP in Sham (circles, n=8), PBS control (squares, n=20), shSCR MSCs (upward triangles, n=19), and shSDF-1 MSCs (downward triangles, n=20). Data are represented as pg/ml. *P*=0.0237 with significant comparison * versus Sham. NS=not significant.

Supplemental Table 1. Antibodies used for flow cytometry cell phenotyping

Antibody	Company	Clone	Catalog number	Configuration
CD105	Biolegend	MJ7/18	120413	APC
CD90.2	eBioscience	30-H12	12-0903-81	PE
CD73	Biolegend	TY/11.8	127210	APC
CD140b	Biolegend	APB5	136007	APC
CD29	eBioscience	HMB1.1	17-0291-80	APC
CD44	eBioscience	IM7	12-0441-82	PE
Sca-1	eBioscience	D7	12-5981-82	PE
CD45	Biolegend	30-F11	103108	FITC
CD11b (mouse)	Biolegend	M1/70	101206	FITC
Lineage cocktail (CD3, Gr-1, CD11b, CD45R/B220, TER-119)	Biolegend	145-2C11, RB6-8C5, M1/70, RA3-6B2,Ter-119	133301	FITC
MHCII (I-A/I-E)	eBioscience	M5/114.15.2	17-5321-82	APC
Ly6G	Biolegend	1A8	127613	APC
Ly6G	Biolegend	1A8	127606	FITC
F4/80	Biolegend	BM8	123108	FITC

SUPPLEMENTAL MATERIALS AND METHODS

Isolation of MSCs. Mouse MSCs were harvested from the compact bone of BALB/c mice as described (1). Lung fibroblasts were used as control mesenchymal cells, or phosphate buffered saline (PBS) as a vehicle. MSCs were grown in MesenCult medium including 10X mouse supplement (STEMCELL Technologies, Cambridge, MA) and 1X Penicillin Streptomycin L-Glutamine (Corning, Manassas, VA). MSCs passages 3 to 6 were used for experiments. The cells were characterized using a BD fluorescent-activated cell sorting (FACS) Canto II (BD Biosciences, Billerica, MA), and analyzed using FlowJo software. Antibodies used for phenotyping of cells are listed in **Supplemental Table 1**.

MSC conditioned medium (CM). Passage 3-6 mouse MSCs were cultured to 80-90% confluence in 10 cm culture dishes, then washed with PBS, and replenished with supplement-free MesenCult medium (STEMCELL Technologies, Cambridge, MA). After 24 hours, the CM was collected, and centrifuged at 2000 rpm for 5 minutes to remove cell debris. The medium was then concentrated using Amicon Ultra-4 centrifugal filter units with 3-kDa cut-off (Millipore, Billerica, MA) following the manufacturer's instructions. Aliquots of the concentrated MSC-CM were then kept at -80°C until its use.

Silencing of SDF-1. For the silencing of SDF-1, target sequence CATCAGTGACGGTAAACCAGTC(consortium number TRCN0000195944), or a scrambled (SCR) sequence, was cloned into the pLKO.1-puro vector (Sigma-Aldrich, Natick, MA), and lentiviral particles were generated using a commercially available packaging mix (Sigma-Aldrich, SHP001) in human embryonic kidney 293 T cells,

according to the manufacturer's instructions. The MSCs were infected with the lentiviral particles, and stably selected by use of puromycin (10 μg/ml) as described (2).

Assessment of SDF-1 silencing by qRT-PCR and ELISA. Total RNA was extracted from shSDF-1 MSCs and shSCR MSCs by TRIzol® reagent, according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). qRT-PCR with SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA) was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) as described (3, 4). The samples were treated with DNase I to degrade any genomic DNA contamination, and cDNA synthesized by iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories). qRT-PCR was performed for SDF-1 using the mouse primers of SDF-1 forward 5'-GAGCCAACGTCAAGCATCTG -3' and reverse 5'-CGGGTCAATGCACACTTGTC-3'. qRT-PCR of β-actin was employed for normalization of SDF-1 expression by the comparative Ct method, using primers of mouse β-actin forward 5'-ACCAACTGGGACGATATGGAGAAGA-3' and reverse 5'-TACGACCAGAGGCATACAGGGACAA-3'. In addition, cell culture supernatants were collected from MSCs, and from plasma of septic mice in each group, and stored at -80°C. Samples were thawed on ice before analysis. SDF-1 was measured in supernatants of cultured MSCs and plasma with a commercially available ELISA kit (R&D, Minneapolis, MN) according to the manufacturer's instructions.

Cecal ligation and puncture (CLP). Polymicrobial sepsis was induced by CLP, as described (1, 5, 6). Briefly, male mice 7-9 weeks of age were anesthetized, the peritoneum

was opened, and two-thirds of the cecum was ligated and punctured with two 21-gauge holes. The cecum was replaced, and 1 ml sterile PBS was injected subcutaneously. In sham experiments, the same procedure was performed, however cecal ligation and puncture were not performed. The mice received MSCs (5 X 10^5 cells/200 µL PBS) or vehicle (PBS 200 µL) via intravenous administration at 2 hours after CLP, and then again at 24 hours after CLP (2.5 X 10^5 cells/200 µL PBS or PBS 200 µL). Depending on the experiment, the mice were either sacrificed at 24 hours after one injection of MSCs, or they were monitored over a 7-day period after two injections of MSCs to determine survival.

Assessment of liver and kidney function, and G-CSF levels. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatinine levels were measured from the plasma at 24 hours after CLP or sham surgery by commercially available colorimetric assay kits (BioVision, Milpitas, CA). For G-CSF analysis, plasma samples were assessed using the multiplex assay technology by Luminex as described previously(3, 7).

Bacterial colony forming units and inflammatory cell analysis of blood and peritoneal lavage. Mice underwent CLP or sham surgery, followed by injection with shSCR or shSDF-1 MSCs two hours later. The mice were sacrificed 24 hours after CLP or sham surgery, and peritoneal lavage was performed followed by phlebotomy of whole blood. The blood and peritoneal fluid was assessed for colony forming units (CFUs) of bacteria, and peritoneal fluid was also evaluated for a differential count of white blood cells. Serial dilutions of whole blood and peritoneal fluid were performed, and then

incubated overnight at 37°C on LB agar plates. CFUs of bacteria were counted and calculated as described (1). Cells from the remaining peritoneal fluid were stained with antibodies targeting Ly6G-APC (BioLegend, Dedham, MA) and F4/80-FITC(BioLegend), to identify neutrophils and macrophages respectively. The cells were then assessed by flow cytometry using a BD FACS Canto II, and analyzed by FlowJo software. A cytospin was also performed on the peritoneal cells, followed by Hema 3 (Fisher Scientific, Hampton, NH) staining. A manual differential count was then carried out by viewers blinded to the groups. The differential count was divided into the predominant cell populations of mature neutrophils, immature myeloid cells (banded neutrophils, myelocytes, and metamyelocytes) and monocytes/macrophages.

Isolation of murine neutrophils. For the isolation of neutrophils, mice were given an intraperitoneal injection of Bio-Gel P100 polyacrylamide beads (2% solution, Bio-Rad Laboratories) as described (1). After 24 hours, the mice were anesthetized and 10 ml of sterile PBS was used to lavage the peritoneal cavity, and cells were washed and placed in Roswell Park Memorial Institute (RPMI) 1640 medium with 0.3% bovine serum albumin and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

MPRO cell line was purchased from ATCC (Manassas, VA). MPRO cells exhibit promyelocyte characteristics, and can be differentiated into immature or mature murine neutrophils using all-transretinoic acid (ATRA) as described.(8, 9) The MPRO cell line was grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 2% fetal bovine serum, 1% penicillin-streptomycin and 10ng/ml recombinant murine GM-CSF

(PeproTech, Rocky Hill, NJ). For differentiation to neutrophils, MPRO cells were treated with 10uM ATRA (Sigma, St Louis, MO) for either 1 day (immature) or 3 days (mature).

Phagocytosis and killing assays. MSCs were added at a ratio of 1 MSC to 5 neutrophils. Isolated neutrophils or MPRO cells were activated with 10 ng/ml of G-CSF (mouse) for 2 hours. Green fluorescent protein (GFP)-labeled *Escherichia coli* (strain MMB1287) or *Enterococcus faecalis* (strain V583) were then added at 10 multiplicity of infection per neutrophil. Bacterial phagocytosis was measured by flow cytometry as described (1, 2). For the killing assay(10), after incubation with bacteria, MPRO cells were harvested and incubated with gentamycin (200 μg/ml) for 30 minutes at 37°C. The cells were then lysed with Triton X-100 (0.1 %), plated on LB-ampicillin agar plates, and incubated overnight at 37°C. The next day CFUs of bacteria were counted and calculated as described (1).

Histology and immunohistochemistry. Mice were sacrificed 24 hours following CLP or sham surgery, and the lungs and femurs were harvested for histological evaluation. The tissues were fixed in 10% formalin, processed, embedded in paraffin, and sectioned (5 μm). Lung tissue was immunostained with a Ly6G (BioLegend) antibody, for assessment of neutrophil infiltration. The area of positively stained cells was calculated per 20X objective using FRIDA Software (FRamework for Image Dataset Analysis, (6)), and numerous random fields were assessed per tissue section. Femurs were processed similar to the lungs, however after fixation the bones were decalcified (0.5M EDTA for 7 days) prior to processing and sectioning. Sections of the femurs where then stained for

H&E to assess cellularity of the bone marrow. The area of cellularity was calculated using ImageJ Software. These sections were also immunostained with a SDF-1 (R&D Systems) antibody, and the area of positively stained cells was calculated as described for Ly6G.

Assessment of cell death in the bone marrow. Terminal deoxynucleotidetransferase–mediated dUTP nick end-labeling (TUNEL) to detect apoptotic cell death (11) in the bone marrow, was performed on tissue sections of femurs from mice of various groups. TUNEL staining was quantified as described above for Ly6G staining. In addition, quantification of neutrophil cell death in the bone marrow of mice was assessed by flow cytometry to determine the percentage of Annexin V-positive cells also staining positive for Ly6G (gating strategy, **Supplemental Figure 8**).

Cell tracking by bioluminescence live imaging, or fluorescent imaging. Non-invasive optical imaging with bioluminescence was used to track MSCs *in vivo* as described (12). MSCs (shSCR and shSDF-1) were infected with firefly luciferase construct (pLenti CMV Puro LUC, Addgene), and lentiviral particles were generated using a commercially available packaging mix (Applied Biological Materials, Inc., Richmond, BC). 5 x 10^5 cells were injected via the tail vein 2 hours after CLP. 36 hours later, the mice were anesthetized and injected with 150 mg/kg RediJect D-Luciferin potassium salt (PerkinElmer, Waltham, MA) intraperitoneally 10 minutes prior to imaging. Photoemission was then measured using the Bruker In-Vivo Xtreme 2 system, Billerica, MA. In addition, scrambled or SDF-1 silenced MSCs were stained with a

PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich) according to the manufacturer's instructions. 2 hours after CLP, PKH26 stained MSCs (shSCR or shSDF-1) or no MSCs were injected intravenously into mice. Bone marrow cells were collected from femurs and tibias at 36h after CLP surgery, and a cytospin of the cells was perform followed by staining with DAPI. The cytospins were then imaged by fluorescent microscopy to assess for the presence of shSCR or shSDF-1 MSCs (red).

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