# Pulmonary Stromal-Derived Factor-1 Expression and Effect on Neutrophil Recruitment during Acute Lung Injury[1]

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**The severe and protracted inflammation that characterizes acute lung injury (ALI) is driven by the ongoing recruitment of neutrophils to the lung. Although much of the cytokine signaling responsible for the initial phase of ALI has been elaborated, relatively little is known about the mechanisms governing the recruitment of neutrophils from the bone marrow to the lung in the later period of this disease. Given its previously described chemoattractant effects on marrow neutrophils, we investigated whether stromal-derived factor-1 (SDF-1) (CXCL12) might participate in this later phase of recruitment. Using immunohistochemistry to examine both banked human lung specimens from patients with ALI and lungs from mice with LPS-induced pneumonitis, we found that pulmonary SDF-1 expression increases during ALI. We further determined that both lung SDF-1 protein expression and mRNA expression rise in a delayed but sustained pattern in this mouse model and that the major source of the increase in expression appears to be the lung epithelium. Lastly, we found that expression of the SDF-1 receptor CXCR4 rises in a similar temporal pattern on neutrophils in both the blood and airspace of LPS-injured mice and that Ab-mediated SDF-1 blockade significantly attenuates late but not early pulmonary neutrophilia in this model. These results implicate SDF-1 in neutrophil recruitment to the lung in the later period of acute lung injury and suggest a novel role for this cytokine in coordinating the transition from the inflammatory response to the initiation of tissue repair.** *The Journal of Immunology,* **2007, 178: 8148–8157.**

Acute lung injury (ALI)[3] is defined in part by persistent, uncontrolled pulmonary inflammation that occurs in response to a variety of insults, including pneumonia, sepsis, and trauma (1). Hence, pulmonary recruitment of neutrophils appears to be a central factor in both the syndrome's onset and its progression (2–4). The neutrophilic response seen in ALI may be considered to occur in two phases: an initial recruitment of neutrophils to the lung after an inciting injury and a second, persistent influx of these cells that further injures the already damaged lung (3, 5–8). Animal and human data suggest that this second or "persistent" phase of pulmonary neutrophilia represents the recruitment of marrow-released neutrophils that manifest a distinct phenotype characterized by increased oxidant production and greater cell stiffness, size, and adherence but decreased chemotaxis and diapedesis to proinflammatory cytokines (9–13). Such a cellular phenotype appears to reflect functional immaturity due to accelerated marrow release (14–16), and has been implicated in the pathophysiology of ALI (13, 16, 17).

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[3] Abbreviations used in this paper: ALI, acute lung injury; BAL, bronchoalveolar lavage; IHC, immunohistochemistry; SDF-1, stromal-derived factor-1.



Animal models and clinical studies have established that injuries inciting ALI result in a rapid induction of proinflammatory cytokines that drive the initial recruitment of neutrophils to the lung (18). Although relevant human data are conflicting (3, 19–22), mouse models of ALI demonstrate that the neutrophil attractant cytokines (such as KC and MIP-2) rapidly taper in both the blood and the lung after initial release despite the ongoing recruitment of neutrophils to the lung (8, 19, 23–27). Thus, although the persistent recruitment of neutrophils in ALI may in part represent a residual effect of early cytokine release, the mechanisms underlying this critical later phase of the disease are unclear. In addition to the attenuation of neutrophil attractant cytokines in the persistent phase of ALI, characteristics of the neutrophilia itself suggest that different mechanisms may drive this process. Neutrophils in the persistent phase of inflammation enter the circulation as a result of greatly accelerated marrow release and, as a consequence, possess a blunted chemoattractant response to traditional neutrophil chemokines (9, 14). Thus, persistent neutrophilia may require not only an alteration in marrow neutrophil retention but alternative pathways of neutrophil chemoattraction to, and diapedesis within, the lung.

One potential chemokine pathway modulating persistent neutrophilia in ALI is the CXCR4/SDF-1 axis. First described in 1996, the CXC chemokine stromal-derived factor-1 (SDF-1) was initially thought to be constitutively expressed in lymphoid organs and the bone marrow and to be critical for hemopoietic development and lymphocyte homing through its cognate receptor CXCR4 (28–30). Subsequently, it has been shown that SDF-1 is both more widely expressed and actively modulated (31). Recent reports have described the pulmonary expression of SDF-1 and have suggested roles for this cytokine in the homing of both malignant metastases and adult stem cells to the lung in mouse models (32–37). Although SDF-1 appears to be up-regulated in an animal model of

lung fibrosis (34, 36), its expression has not been examined in the setting of ALI.

Our group and others have previously demonstrated that SDF-1 is a chemoattractant for neutrophils within the bone marrow and is responsible for marrow retention and marrow homing of these cells under homeostatic conditions (29, 38–40). How the CXCR4/SDF-1 axis might participate in the development of inflammation is poorly understood, but several reports support a role for this pathway in the tissue migration of leukocytes, including neutrophils, in animal models of arthritis (41–44) and peritonitis (45). A role for SDF-1 in lung inflammation has been suggested by animal models of asthma in which a CXCR4 blockade attenuates both the lymphocyte and eosinophil responses and reduces airway hyperreactivity (46, 47). However, no investigations of the CXCR4/SDF-1 axis in the recruitment of neutrophils to the lung have been reported, and whether pulmonary SDF-1 expression occurs during the development and progression of ALI is unknown. We investigated pulmonary SDF-1 expression during ALI in mice and humans, and examined the potential role of SDF-1 in neutrophil recruitment using a mouse model of pneumonitis.

## Materials and Methods

### *Mice*

Four- to eight-week-old female C57BL/6 mice were obtained from Harlan and housed in the animal facilities of the University of Vermont College of Medicine. All experiments were performed in accordance with the Animal Welfare Act and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals after review of the protocol by the Animal Care and Use Committee of the University of Vermont (Burlington, VT).

### *Reagents*

Mouse anti-human SDF-1 mAb (clone 79018; R&D Systems), rabbit polyclonal anti-cytokeratin (catalog no. 18-0059, Zymed Laboratories), Alexa Fluor 568-labeled goat anti-mouse $F(ab')_2$ (catalog no. A11019; Invitrogen Life Technologies), and Alexa Fluor 488-labeled donkey anti-rabbit IgG (catalog no. A21206; Invitrogen Life Technologies) were purchased for use in immunohistology. Biotinylated rat anti-mouse CXCR4 mAb (2B11/CXCR4) and control (rat IgG2b) were obtained from BD Pharmingen for use in flow cytometry. Rat anti-mouse CD16/CD32 and FITC-conjugated rat anti-mouse Ly-6G (Gr-1) mAbs were also obtained from BD Pharmingen. LPS derived from *Escherichia coli* 0111:B4 was purchased from Sigma-Aldrich. Mouse anti-mouse/human SDF-1 mAb (clone 79014) and control Ab (mouse IgG1) for use in in vivo blocking experiments were obtained from R&D Systems. The 50% neutralizing dose ($ND_{50}$) of this Ab is reported to be from 30 to 100 $\mu$g/ml depending on the cell type and activity measured in vitro (R&D Systems), and the Ab is specific for CXCL12/SDF-1 in both in vivo and in vitro assays examining murine CXC and CC cytokines (R&D Systems). Additional studies in our lab using direct ELISA demonstrated no binding of this Ab to murine TNF-$\alpha$ or IL-1$\beta$.

### *LPS pneumonitis*

Mice were exposed to aerosolized LPS for 15 min in a modified Tepper box (Genentech) using a 3 mg/ml solution of LPS nebulized by a Pari LC Plus reusable nebulizer with ProNeb turbo air compressor (Pari Respiratory Equipment). The animals were euthanized 3–4 h later by pentobarbital overdose. Blood was collected via cardiac puncture into a syringe containing EDTA and 1× protease inhibitor mixture (catalog no. P 8340; Sigma-Aldrich). An aliquot was analyzed for cell count and differential using an Advia 120 hematology analyzer with veterinary software (Bayer), and serum from the remaining blood was frozen for later analysis. Bronchoalveolar lavage (BAL) was performed as previously described (48). Briefly, a midline neck incision was performed and an 18-gauge catheter (Baxter Health Care) was inserted into the trachea and secured with sutures. Lungs were lavaged with 1 ml of chilled 0.1% BSA in PBS with 1× protease inhibitor mixture. The BAL was spun for 6 min at 500 × *g* and the resulting supernatant was aspirated and snap frozen for later analysis while the cell pellet was resuspended in 5% BSA in PBS and analyzed for cell count and differential using the Advia 120, and cellular SDF-1 protein and mRNA were determined, as described below.

### *Immunohistology*

Banked human lung specimens were reviewed from patients who had previously undergone a videoscopic biopsy for the diagnostic evaluation of diffuse lung injury with respiratory failure and who were found to have histological evidence of diffuse alveolar damage (the histological correlate of ALI). Banked specimens were also reviewed from patients who underwent a videoscopic lung biopsy for the evaluation of solitary lung nodule and who were found to have histologically normal lung tissue without evidence of injury, infection, or malignancy. All lung specimens were reviewed by an expert pulmonary pathologist, and three specimens were chosen for SDF-1 immunohistochemistry from both the ALI group and the normal group. All patient specimens were examined in accordance with an approved Institutional Review Board protocol. Five-micrometer sections of the selected formalin-fixed, paraffin-embedded specimens were prepared and SDF-1 staining was performed using an anti-human SDF-1 Ab (1/100; clone 79018, R&D Systems) with a peroxidase-conjugated secondary Ab followed by 3,3′-diaminobenzidine (Sigma-Aldrich). Appropriate IgG control stains were also performed. Stained slides were examined by light microscopy with a Zeiss Axioskop 2 Plus microscope with an Axiocam HRc digital camera, and representative ×200 images were chosen from each group (control and ALI).

Whole murine lungs from either LPS- or saline-treated animals (24 h after exposure) were prepared and stained for SDF-1 as follows. A midline neck incision was performed and an 18-gauge catheter was inserted into the trachea and secured with sutures. The lungs were then dissected from the thoracic cavity and instilled via the catheter with 4% paraformaldehyde to 25-cm water pressure. The trachea was then tied and the lungs were submerged in 4% paraformaldehyde at room temperature for 24 h. The fixed lungs were embedded in paraffin and 5-$\mu$m slices were mounted on slides before SDF-1 staining was performed using anti-SDF-1 Ab (1/100; clone 79018, R&D Systems) and a mouse-on-mouse immunodetection technique (M.O.M. kit; Vector Laboratories) with a peroxidase-conjugated secondary Ab. The slides were then developed with 3,3′-diaminobenzidine. Appropriate IgG control stains were also performed. Four animals were studied for each condition and stained lung sections were examined by light microscopy as above. Representative ×200 images were chosen for each condition.

Disaggregated murine lungs from either LPS- or saline-treated animals (prepared as detailed below) were analyzed by immunofluorescent staining as follows. Cytospins were prepared of both leukocyte ($CD45^+$) and structural ($CD45^-$) lung cell fractions and fixed with 4% paraformaldehyde in PBS before being washed with cold PBS and incubated in 100% methanol at −20°C for 10 min. The fixed cells were then treated with 0.1% Triton X-100 in PBS (15 min at room temperature), rinsed with PBS, and then blocked with 10% goat serum (Vector Laboratories) in PBS (60 min at room temperature). The slides were next incubated with a combination of murine anti-SDF-1 (1/200 in PBS and 1% BSA; clone 79018, R&D Systems) and rabbit polyclonal anti-cytokeratin (1/400; catalog no. 18-0059, Zymed Laboratories) for 60 min at room temperature in a humidified chamber before being washed twice with PBS and then incubated with a combination of Alexa Fluor 568 goat anti-mouse $F(ab')_2$ (1/400; catalog no. A11019, Invitrogen Life Technologies) and Alexa Fluor 488-labeled donkey anti-rabbit IgG (1/400; catalog no. A21206, Invitrogen Life Technologies) for 30 min. The slides were then counterstained with 4′,6′-diamidino-2-phenylindole, washed, coverslipped, and visualized using a Zeiss LSM 510 META confocal scanning laser microscope (Carl Zeiss MicroImaging) with LSM Version 3.2 software.

### *SDF-1 protein quantitation*

Frozen mouse lung samples and cell pellets (from BAL and cell culture) were thawed in PBS with 1× protease inhibitor mixture (catalog no. P 8340, Sigma-Aldrich) and homogenized on ice using a Polytron PT2100 (Kinematica). Homogenates were sonicated using a Microson ultrasonic cell disruptor (model no. XL2005, Heat Systems Ultrasonics) and then centrifuged for 10 min at 2000 × *g*. The resulting supernatants were carefully aspirated and the total protein concentrations were determined by a Bradford assay. Supernatants were then assayed for SDF-1 protein levels using the Quantikine mouse CXCL12/SDF-1$\alpha$ ELISA kit (catalog no. MCX120, R&D Systems). Results are reported as picograms of SDF-1 per milligram of total protein. Serum and BAL supernatant and cells, collected as detailed above, were analyzed for SDF-1 protein using the Quantikine mouse CXCL12/SDF-1$\alpha$ ELISA kit. Six animals from three separate experiments were analyzed for each time point.

### *Quantitative RT-PCR for tissue SDF-1 mRNA*

Frozen mouse lung samples, obtained as detailed above, were pulverized using chilled mortars and pestles. Cells from BAL and cell culture,

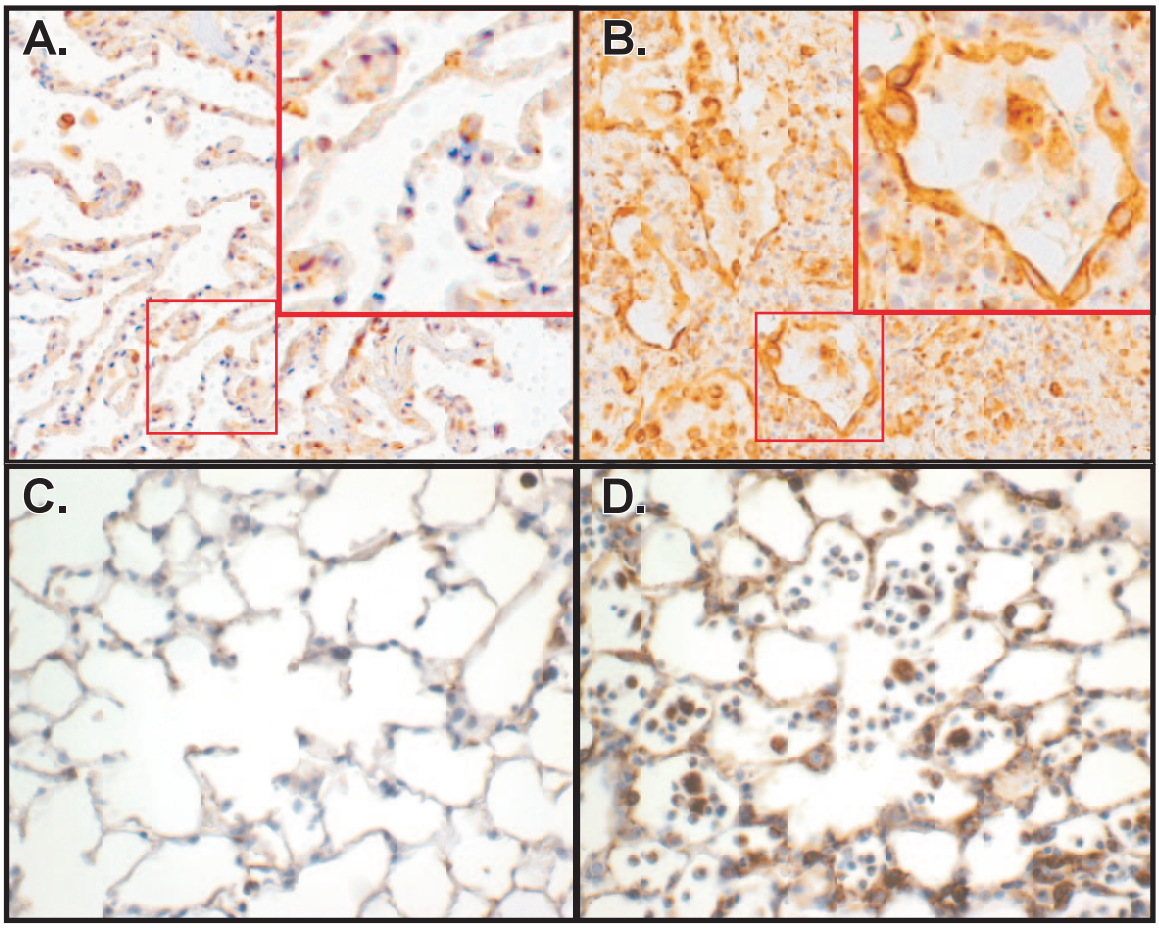


**FIGURE 1.** Immunohistochemical evidence of increased SDF-1 expression in the lungs of patients and mice with ALI. Shown are a normal lung (*A*) and a lung from a patient with histological evidence of ALI (*B*). *Insets* represent blow-ups of framed areas. Brown staining indicates SDF-1. Representative images are shown from normal control ($n = 3$) and ALI ($n = 3$) biopsy samples (see *Materials and Methods*). Also shown are normal mouse lung (*C*) and LPS-exposed mouse lung at 24 h (*D*). Representative images are shown from control ($n = 4$) and LPS-exposed mice ($n = 4$) (see *Materials and Methods*). Original magnification, ×200 (*insets* approximately ×400).

obtained as detailed above, were pelleted by centrifugation. RNA was extracted using TRIzol and treated with DNase using RNEasy columns (Qiagen), and 1.0 $\mu$g of total RNA was used as a template to synthesize first-strand cDNA using random primers and SuperScript II reverse transcriptase mix according to instructions by the manufacturer (Invitrogen Life Technologies). Real-time semiquantitative RT-PCR was performed using the TaqMan universal PCR master mix and the ABI PRISM 7700 sequence detection system. The probes and primer sets used for mouse hypoxanthine guanine phosphoribosyl transferase (*Hprt*) were 5′-TTTGCC GCGAGCCG-3′ (forward), 5′-TAACCTGGTTCATCATCGCTAATC-3′ (reverse), and 5′-FAM-CGACCCGCAGTCCCAGCGTC-BHQ-1-3′ (probe), and for *SDF-1* the sets were 5′-CCCATTCTCCTCATCCTCAT-3′ (forward), 5′-ACTCTGCTCTGGTGGAAGGT-3′ (reverse), and 5′-FAM-TCTTTGCC CTGGGGCCTCTG-BHQ-1-3′ (probe); all were purchased from Biosearch Technologies. *SDF-1* levels were analyzed using the cycle threshold ($\Delta\Delta$Ct) method and normalized to hypoxanthine guanine phosphoribosyl transferase. Tissue *SDF-1* mRNA expression for injured lungs was reported as the fold increase over uninjured mouse lung tissue expression. Six animals from three separate experiments were analyzed for each time point.

### *Alveolar macrophage culture and LPS stimulation*

Murine alveolar macrophages were obtained from five mice by alveolar lavage and cultured in MEM with 10% FCS at a density of $2.5 \times 10^5$ cells/well (24-well plate) as previously described (49). After 24 h in culture, macrophages were stimulated by adding LPS to the medium in a dose-response curve (final concentrations 0, 0.1, 0.25, 0.5, and 1.0 $\mu$g/ml) followed by incubation for 24 h. After incubation, cell-free medium was aspirated and frozen (for SDF-1 protein assay) and macrophages were lysed for mRNA by adding TRIzol directly to the aspirated wells with subsequent analysis by quantitative RT-PCR as detailed above.

### *Lung disaggregation and magnetic bead separation*

Murine lungs from either LPS- or saline-treated animals (24 h after exposure) were disaggregated and separated into leukocyte ($CD45^+$) and structural ($CD45^-$) cellular fractions as follows. After euthanasia the trachea was cannulated as described above (without lavage being performed), 1 ml of DMEM containing DNase I (200 $\mu$g/ml; Invitrogen Life Technologies) and type 1 collagenase (1 mg/ml; Invitrogen Life Technologies) was instilled into the lungs, and the pulmonary vascular system was perfused with cold PBS via needle puncture of the right ventricle. The lungs were then removed, surgically minced, and shaken at 37°C for 15 min in DMEM/DNase/collagenase solution before further mechanical disruption using a 15-gauge blunt metal needle. The cell solution was passed through a 70-$\mu$m strainer, washed with cold PBS, and treated with Geye's solution to induce RBC lysis before being washed with DMEM twice (at 300 × *g* for 5 min at 4°C) and resuspended in MACS buffer (PBS with 0.5% BSA and 2 mM EDTA) at a concentration of $1.0 \times 10^9$ cells/ml. Ninety-microliter aliquots of cells were next incubated with 10 $\mu$l of anti-CD45 magnetic microbeads (Miltenyi Biotec) for 15 min at 4°C and then washed twice with MACS buffer before being loaded onto a MACS MS column as directed by the manufacturer (Miltenyi Biotec) and eluted as $CD45^+$ and $CD45^-$ cell fractions. Isolated cells were then analyzed for *SDF-1* mRNA by quantitative PCR or SDF-1 protein by immunofluorescent staining as described above. Five animals from three separate experiments were analyzed.

### *Determination of neutrophil surface CXCR4 expression*

Murine blood and BAL neutrophils ($1 \times 10^5$), isolated from LPS-treated mice as detailed above, were resuspended in staining buffer (PBS with 5% FCS) and incubated with anti-CD16/CD32 mAbs for 30 min at 4°C. The

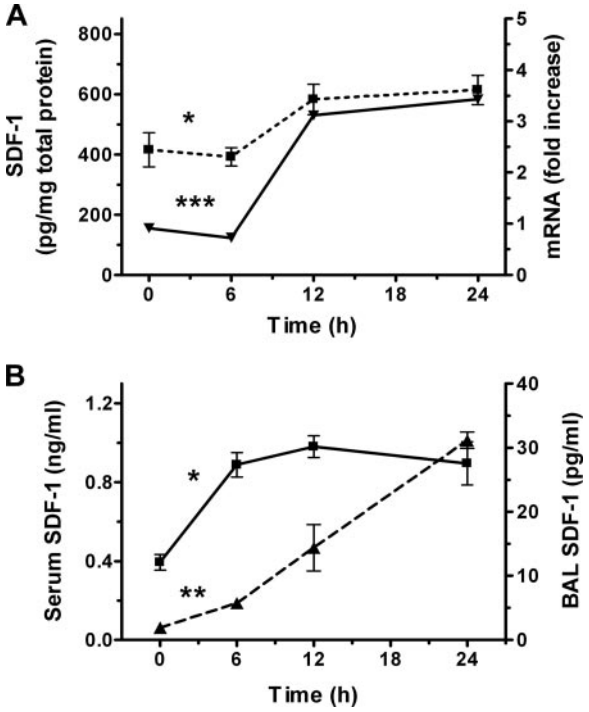


**FIGURE 2.** The time course of SDF-1 protein and mRNA induction in murine lung during LPS injury shows delayed yet sustained up-regulation. Mice were exposed to inhaled LPS and at the indicated times were euthanized for analysis. *A*, Total protein (dashed line) was isolated from homogenized, sonicated whole lung, assayed by SDF-1 ELISA, and reported as picograms of SDF-1 per milligram of total lung protein. Lung SDF-1 mRNA (solid line) was determined by quantitative RT-PCR and expressed as the fold-increase over uninjured (0 h) mice. *B*, Serum (solid line) and BAL (dashed line) levels of SDF-1 protein; $n = 6$ mice per time point. *, $p = 0.02$, significant SDF-1 protein increase over time by one-way ANOVA; **, $p < 0.0001$, significant SDF-1 mRNA increase over time by one-way ANOVA.

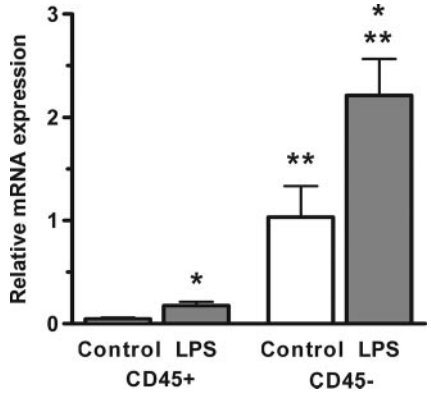


**FIGURE 3.** SDF-1 mRNA expression in LPS-injured mice occurs primarily in the structural (nonleukocyte) compartment of the lung. Control and LPS-injured murine lungs (24 h) were disaggregated and the total cells were sorted by magnetic bead separation into $CD45^+$ (leukocyte) and $CD45^-$ (structural) compartments before analysis by quantitative PCR for *SDF-1* mRNA (see *Materials and Methods*). In both control and injured lungs the predominant source of the SDF-1 transcript is the $CD45^-$ compartment (comprised of epithelium, fibroblasts, and endothelium), whereas a small amount is found in pulmonary leukocytes ($CD45^+$). Induction of *SDF-1* mRNA occurs in both compartments following LPS injury; $n = 5$ mice/condition. Data were normalized to control $CD45^-$ mRNA content. *, $p < 0.05$, *SDF-1* mRNA content greater compared with respective control; **, $p < 0.01$, *SDF-1* mRNA content greater compared with $CD45^+$ control and injured cells.

cells were then stained sequentially with anti-Gr-1-FITC mAb, biotinylated anti-CXCR4 or isotype control mAbs, and then streptavidin-PE, each for 30 min at 4°C. Cells were examined using a FACScan flow cytometer (BD Biosciences) and data were analyzed by FlowJo software (Tree Star). Neutrophils were identified by gating for $Gr\text{-}1^{high}$ cells, and this population was then analyzed for CXCR4 expression. Three to five separate experiments were performed for each analyzed population of neutrophils. Results were expressed as a ratio of the mean fluorescence intensity of cells stained with the CXCR4 Ab vs the isotype control Ab. Representative flow histograms are also presented.

### *Murine blood neutrophil chemotaxis to SDF-1*

A modified Boyden chamber assay to examine the neutrophil chemoattractant response to SDF-1 was performed using a 48-well microchamber (Neuro Probe). Murine blood neutrophils were isolated as previously described (48) and resuspended in H/H running buffer (1× HBSS, 2 mg/ml BSA, 10 mM HEPES, 1 mM $CaCl_2$, and 1 mM $MgCl_2$). Jurkat (acute lymphoblastic leukemia) cells were cultured as described (50) to serve as a positive control in the assay. Recombinant mouse CXCL-12/SDF-1$\alpha$ (50 ng/ml; R&D Systems) in H/H buffer (for wells containing neutrophils), basal medium (for wells containing Jurkat cells), or appropriate buffer control were added to the lower chambers of the apparatus. A 6-$\mu$m pore polycarbonate membrane (Neuro Probe) was placed between the upper and lower chambers, and $5 \times 10^4$ cells in a volume of 50 $\mu$l were added to the top chambers of the apparatus. Cells were allowed to migrate into the membrane for 1 or 2 h per treatment at 37°C with 5% $CO_2$. All control wells were incubated for 2 h. Following incubation, the chamber was disassembled and the membrane was scraped and washed three times in PBS to remove nonadherent cells before being fixed in methanol and stained using the Diff-Quik system (Dade Behring). Each well-associated membrane area was scored using light microscopy to count the intact cells present. In a similar set of studies, the effect of SDF-1 blocking Ab (10 $\mu$g/well; clone 79014, R&D Systems) on neutrophil chemotaxis to SDF-1 at 2 h was analyzed compared with an isotype control Ab.

### *SDF-1 blockade and augmentation during LPS pneumonitis*

In the initial experiments mice were injected with SDF-1 blocking Ab (50 $\mu$g IV, clone 79014, R&D Systems) or isotype control Ab, immediately exposed to nebulized LPS, and thereafter euthanized for blood and BAL analysis 24 h later (as described above). Subsequent experiments used an intratracheal approach as follows. LPS (10 $\mu$g) and SDF-1 blocking Ab (50 $\mu$g) or isotype control Ab (50 $\mu$g) were combined in a sterile 0.9% NaCl solution to a final volume of 50 $\mu$l. Mice were anesthetized with inhaled isoflurane and suspended by their incisors on a 60° incline board. The tongue was gently extended, and the LPS/antibody mixture was delivered into the distal part of the oropharynx with a pipette and aspirated into the lower respiratory tract. Mice were then allowed to aspirate the material for ~15 s, after which they were returned to the cage and allowed to recover from anesthesia (51). Treated and control mice were euthanized 6 or 24 h later, and blood and BAL were assayed as described above. In similar studies, the effects of intratracheal SDF-1 augmentation during LPS pneumonitis were examined through the addition of recombinant murine SDF-1 (10 $\mu$g) or carrier (PBS with 0.1% BSA) to the instilled LPS. Five to seven animals were assayed for each time point and condition in three or four separate experiments.

### *Statistical analysis*

An analysis of differences in serum, BAL, and total lung SDF-1 protein and mRNA at selected time points was performed by one-way ANOVA using Prism 4 software (GraphPad Software). All other comparisons were performed with the Student *t* test using Prism 4 software.

## Results

### *SDF-1 expression increases in human and murine lungs during ALI*

We hypothesized that SDF-1 is up-regulated in the lung during acute lung injury and that it is responsible for the recruitment of neutrophils in the late inflammatory phase. Few reports examining the expression of SDF-1 in the lungs of mice have been published (32, 34, 46), and none used immunohistochemistry

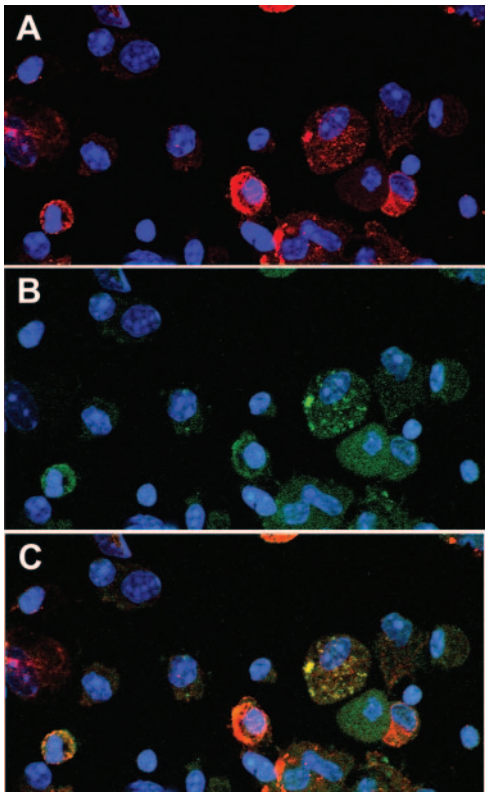


**FIGURE 4.** SDF-1 protein localizes to the pulmonary epithelium by dual-fluorescence immunohistology. $CD45^-$ cells isolated from LPS-injured murine lungs by tissue disaggregation and magnetic bead sorting were stained with both anti-SDF-1 (red in *A*) and anti-pancytokeratin (green in *B*) Abs and counterstained with 4′,6′-diamidino-2-phenylindole (blue). Strong SDF-1 staining is seen in many of the $CD45^-$ cells (*A*), and this staining colocalizes with cytokeratin (*C*; merged image), indicating that the pulmonary epithelium is the source of SDF-1 following LPS-induced lung injury. Representative images are shown ($n = 5$) (see *Materials and Methods*). Original magnification, ×400.

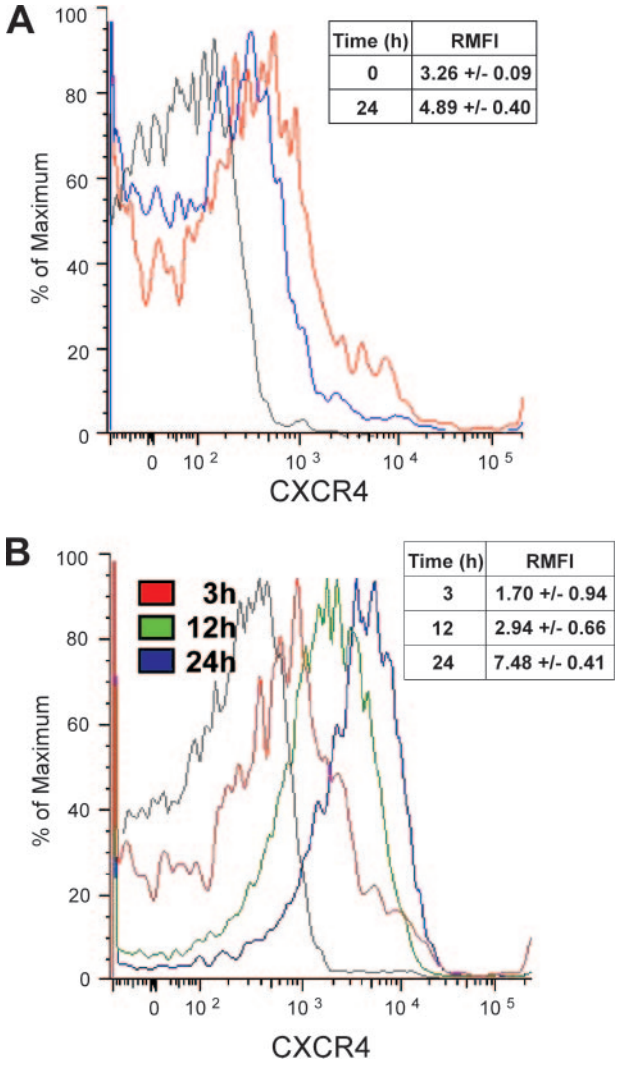


**FIGURE 5.** Increasing levels of CXCR4 expression are found on circulating and airspace neutrophils during the progression of LPS lung injury. Blood and BAL neutrophils were isolated from mice with LPS-induced lung injury at the indicated time points, stained with anti-CXCR4 Ab, and analyzed by flow cytometry (see *Materials and Methods*). *A*, Blood neutrophils demonstrate a small but significant ($p = 0.007$) increase in surface levels of the SDF-1 receptor CXCR4 24 h after the induction of LPS pneumonitis (red) compared with control (blue). *B*, BAL neutrophils assayed at 3, 6, and 24 h after LPS injury show increasing levels of CXCR4 expression over time ($p = 0.002$, by ANOVA). Although the mean rise in blood neutrophil CXCR4 staining is only ~50%, this appears to be due to an increase in a $CXCR4^{high}$ subpopulation that has a signal intensity similar to that seen on airspace neutrophils at 24 h. Gray lines indicate isotype controls. RMFI, Relative mean fluorescence intensity.

(IHC) to examine the lung parenchyma during injury. In addition, SDF-1 expression in human ung tissue has never been described. We examined SDF-1 expression by IHC both in human lung samples from patients with histologic evidence of ALI and in murine lungs 24 h after injury with nebulized LPS as a model of ALI. Limited staining is present in both normal (uninjured) human (Fig. 1*A*) and mouse (Fig. 1*C*) lungs, primarily in the alveolar epithelium and possibly alveolar macrophages. Following the development of injury, both human (Fig. 1*B*) and mouse (Fig. 1*D*) lungs show markedly increased SDF-1 staining. Staining appears to become diffuse during ALI, involving both the alveolar epithelium (particularly what appear morphologically to be type II cells) and the interstitium. Murine tissue staining was performed using the same anti-SDF-1 Ab as that used on human tissues (clone 79018, R&D Systems) and shows a more limited but similar SDF-1 staining pattern compared with the human ALI-affected lung. These findings indicate increased SDF-1 expression in both human and murine lungs during acute lung injury. However, the time course and sources of SDF-1 release are unclear from these studies.

### *Murine pulmonary SDF-1 mRNA and protein rise and plateau during LPS-induced lung injury*

Given the apparent up-regulation of SDF-1 in ALI suggested by IHC, we sought to confirm this finding and elaborate the time course of pulmonary SDF-1 induction during lung injury by examining whole lung *SDF-1* mRNA and protein in mice following the exposure of mice to inhaled LPS. As shown in Fig. 2*A*, normal lung samples ($t = 0$) demonstrate a basal level of SDF-1 protein expression consistent with our IHC findings (Fig. 1). Both *SDF-1* mRNA and protein rise at 12 h and plateau between 12 and 24 h. BAL and serum levels of SDF-1 were found to rise in a similar fashion (Fig. 2*B*). The temporal pattern of SDF-1 expression in the injured lung is distinctly delayed compared with that seen for most previously examined inflammatory cytokines, including other CXC cytokines such KC and MIP-2 (8, 19, 23–27).

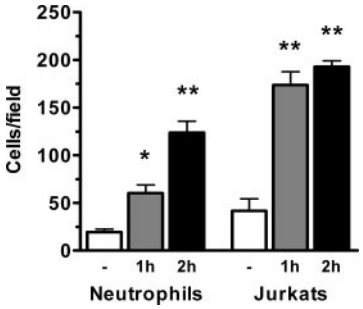


**FIGURE 6.** Murine blood neutrophils display a chemoattractant response to SDF-1. Murine peripheral blood neutrophils were isolated and their chemotaxis to recombinant murine SDF-1 (50 ng/ml) was examined at 1 and 2 h of incubation in modified Boyden chambers. Significant incremental response is seen in response to SDF-1 compared with buffer control (□). The response is somewhat less than but similar to that seen from positive control cells in the assay (Jurkat acute lymphoblastic leukemia cells). *, $p = 0.01$, greater than respective control; **, $p < 0.001$, greater than respective control.

### *Pulmonary epithelium appears to be the primary source of SDF-1 in the injured lung*

To identify the sources of SDF-1 release in the setting of acute lung injury, we first examined airspace leukocytes from LPS-injured murine lungs for SDF-1 protein and mRNA. Although low levels of SDF-1 protein (12.2 ± 1.6 pg/mg total protein) were found in BAL leukocytes (predominantly neutrophils) 24 h after injury, *SDF-1* mRNA levels were undetectable. Similarly, an examination of cultured murine alveolar macrophages also yielded undetectable levels of *SDF-1* mRNA even after LPS stimulation for 24 h. To further investigate the possible sources of SDF-1 production in the injured lung, we next used tissue disaggregation and magnetic bead separation to fractionate the lungs into leukocyte and structural compartments. We found that $CD45^-$ cells in the lung (comprised of epithelium, endothelium, and fibroblasts) appear to be the major sources of SDF-1 in both naive and LPS-injured lung, whereas $CD45^+$ cells (leukocytes) provide a relatively minor contribution (Fig. 3). Further analysis of the $CD45^-$ cell population by dual-fluorescence immunohistology reveals that the vast majority of cells staining for SDF-1 costained for the epithelial marker cytokeratin (Fig. 4). Thus, the primary source of pulmonary SDF-1 release following LPS injury appears to be the epithelium, with a very small component being derived from leukocytes.

It is notable that an analysis of cells isolated from the lavageable airspace of naive and injured mice revealed undetectable levels of SDF-1 mRNA, yet low levels of transcript are seen in the lung disaggregation experiments. This implies that the small leukocyte ($CD45^+$) contribution to total lung SDF-1 mRNA found by lung disaggregation is derived from leukocytes in the vasculature or interstitium of the lung or, conversely, may reflect low level $CD45^-$ cell contamination of the $CD45^+$ fraction in the absence of true leukocyte SDF-1 production. In light of these findings, the SDF-1 staining of cells resembling alveolar macrophages (Fig. 1) may represent association and/or internalization of released cytokine with (or within) the cells present in the airspace.

### *Neutrophils isolated from the blood and airspace of LPS-injured mice show greater levels of surface CXCR4 expression late in the course of injury*

To investigate the role of pulmonary SDF-1 expression in neutrophil recruitment, we next examined the expression of the SDF-1 receptor CXCR4 on blood and airspace neutrophils during the development of LPS pneumonitis in mice. A small but significant ($p = 0.007$) increase in CXCR4 expression was found on circulating neutrophils 24 h after LPS exposure (Fig. 5*A*) but was not evident at earlier time points (data not shown). CXCR4 levels on airspace neutrophils, however, were found to steadily increase over time following lung injury (Fig. 5*B*; $p = 0.002$), suggesting that airspace neutrophilia later in the course of injury is augmented by selective recruitment of $CXCR4^{high}$ neutrophils from the blood.

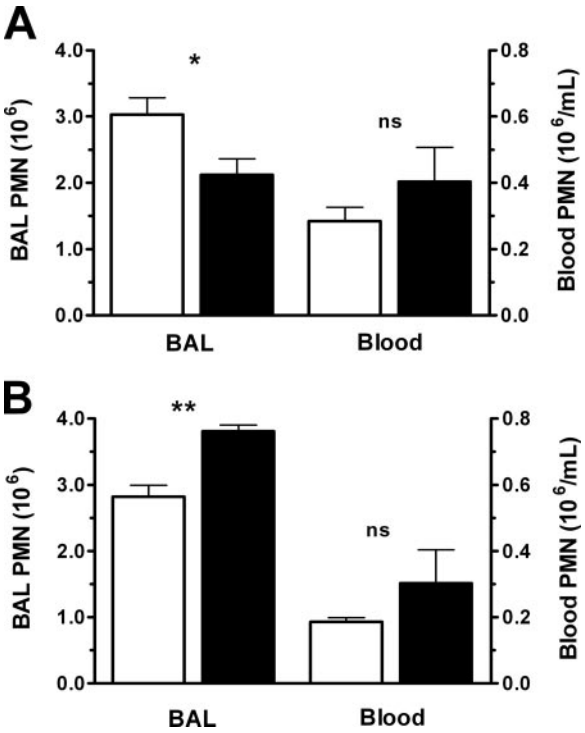


**FIGURE 7.** SDF-1 blockade attenuates and SDF-1 augmentation increases airspace neutrophilia at 24 h after LPS injury. *A*, Mice were treated with LPS and SDF-1 blocking Ab (■) or control Ab (□) by oropharyngeal aspiration following isoflurane anesthesia, then euthanized, and BAL and blood were assayed 24 h later. *B*, In similar experiments, mice were treated with LPS and 10 μg of recombinant murine SDF-1 (■) or vehicle control (□) by oropharyngeal aspiration; $n = 5$–7 mice/time point. *, $p = 0.02$; **, $p < 0.01$; ns, not significant. PMN, Polymophonuclear leukocyte.

### *SDF-1 is a chemoattractant for isolated murine blood neutrophils*

To initially address whether neutrophils might be recruited to the lung by SDF-1, we examined isolated blood neutrophils in chemotaxis experiments using a modified Boyden chamber and recombinant murine SDF-1. As shown in Fig. 6, isolated neutrophils show a significant chemotactic response to SDF-1 that, at 2 h, is nearly 70% that of Jurkat cells (a cell type known to be highly responsive to SDF-1 (52). Ab-mediated blockade of SDF-1 in this assay led to near-complete abrogation of neutrophil chemotaxis (93% reduction, $p = 0.02$).

### *Lung SDF-1 is necessary and sufficient to augment pulmonary neutrophilia in LPS-induced pneumonitis*

We further examined whether pulmonary SDF-1 expression following injury participates in the recruitment of neutrophils to the lung by assessing the effects of SDF-1 blockade and augmentation in mice with LPS-induced pneumonitis. Efforts to block SDF-1 systemically though an i.v. injection of SDF-1 Ab resulted in increased blood neutrophilia in the pneumonitis model but little

Table I. *SDF-1 blockade in vivo attenuates airspace neutrophilia at 24 h after LPS injury but has little effect at 6 h*[a]

| | Six Hours | | | |
|---|---|---|---|---|
| | WBC ($\times 10^6$) | PMN (%) | Mac (%) | Lymph (%) |
| Anti-SDF-1 | 0.59 ± 0.10 | 82.02 ± 2.22 | 16.18 ± 2.22 | 1.80 ± 0.35 |
| Isotype Control | 0.54 ± 0.16 | 73.60 ± 5.68 | 23.08 ± 5.37 | 3.33 ± 0.59 |
| Significance | NS | NS | NS | * |
| | | ABS PMN | ABS Mac | ABS Lymph |
| Anti-SDF-1 | | 0.49 ± 0.10 | 0.09 ± 0.01 | 0.01 ± 0.00 |
| Isotype Control | | 0.42 ± 0.15 | 0.11 ± 0.01 | 0.02 ± 0.00 |
| Significance | | NS | NS | NS |
| | Twenty-four Hours | | | |
| | WBC ($\times 10^6$) | PMN (%) | Mac (%) | Lymph (%) |
| Anti-SDF-1 | 2.35 ± 0.24 | 89.85 ± 1.28 | 9.48 ± 1.31 | 0.67 ± 0.20 |
| Isotype Control | 3.27 ± 0.27 | 92.59 ± 0.45 | 6.97 ± 0.36 | 0.44 ± 0.15 |
| Significance | ** | * | * | NS |
| | | ABS PMN | ABS Mac | ABS Lymph |
| Anti-SDF-1 | | 2.13 ± 0.24 | 0.21 ± 0.01 | 0.02 ± 0.01 |
| Isotype Control | | 3.03 ± 0.25 | 0.23 ± 0.02 | 0.01 ± 0.01 |
| Significance | | ** | NS | NS |

[a] Mice were treated with LPS and SDF-1 blocking Ab or control Ab by oropharyngeal aspiration following isoflurane anesthesia, euthanized 6 or 24 h later, and BAL assayed; $n = 5$–7 mice/time point. Differences between blocking Ab and control: *, $0.09 > p > 0.05$; **, $p = 0.02$; NS, not significant. WBC, White blood cell; ABS, absolute number; PMN, polymorphonuclear leukocyte; Mac, macrophage; Lymph, lymphocyte.

change in BAL neutrophil content at 24 h compared with control (data not shown). The finding of increased blood neutrophilia was expected based on our previous examination of systemic CXCR4/SDF-1 blockade (38). Given that the systemic induction of neutrophilia has been shown to augment neutrophil recruitment to the inflamed lung (53, 54), we considered the possibility that this phenomenon might be confounding our BAL results. We therefore chose to administer the SDF-1-blocking Ab intratracheally to avoid the systemic effects of this Ab and to directly block pulmonary SDF-1, which appears to be predominantly airspace derived.

As shown in Fig. 7*A*, by using the intratracheal approach we found that a SDF-1 blockade significantly attenuated neutrophil influx into the airspace following LPS exposure (a 30% reduction; blocked, $2.13 \pm 0.23 \times 10^6$ vs control, $3.03 \pm 0.25 \times 10^6$ cells total; $p = 0.02$). Full differentials of the BAL fluid from SDF-1-blocked and control mice (Table I; 24 h) demonstrate that the absolute leukocyte and neutrophil counts are significantly reduced in the blocked mice, and a trend ($p = 0.05$) is seen toward a lower percentage of neutrophils and higher percentage of macrophages. An analysis of BAL neutrophil CXCR4 expression in the blocked mice revealed a 19.9% reduction in CXCR4 mean fluorescence compared with control (relative mean fluorescence intensity 7.48 vs 5.99, $p = 0.02$), consistent with a decreased recruitment of $CXCR4^{high}$ neutrophils. To further investigate the role of SDF-1 in pulmonary neutrophil recruitment during ALI, intratracheal instillation of recombinant SDF-1 was also examined in the oropharyngeal LPS model. SDF-1 augmentation in this model led to a significant increase in airspace neutrophilia at 24 h as shown in Fig. 7*B* and Table II. Therefore, SDF-1 appears to play a causal role in the recruitment of neutrophils to the lung in response to LPS injury.

Although not statistically significant, increased blood neutrophilia was also noted in the intratracheally blocked animals (Fig. 7*A*) (blocked, $0.40 \pm 0.10 \times 10^6$ vs control, $0.28 \pm 0.04 \times 10^6$ cells/ml blood, $p = 0.28$) and does not appear to be secondary to systemic effects of the Ab, as intratracheal Ab blockade in the absence of LPS exposure did not alter blood neutrophil levels (data not shown). This finding may suggest that local tissue blockade of SDF-1 leads to blood neutrophilia via the inhibition of neutrophil departure from the circulation and into the lung.

Table II. *SDF-1 augmentation* in vivo *increases airspace neutrophilia at 24 h after /LPS injury*[a]

| | WBC ($\times 10^6$) | PMN (%) | Mac (%) | Lymph (%) |
|---|---|---|---|---|
| SDF-1 | 4.12 ± 0.11 | 92.47 ± 1.08 | 3.60 ± 0.65 | 3.93 ± 1.19 |
| Buffer Control | 3.11 ± 0.23 | 91.13 ± 1.45 | 6.97 ± 0.98 | 1.90 ± 0.67 |
| significance | ** | NS | * | NS |
| | | ABS PMN | ABS Mac | ABS Lymph |
| SDF-1 | | 3.81 ± 0.09 | 0.15 ± 0.02 | 0.16 ± 0.05 |
| Buffer Control | | 2.84 ± 0.22 | 0.21 ± 0.02 | 0.10 ± 0.59 |
| Significance | | * | NS | NS |

[a] Mice were treated with LPS and either SDF-1 (10 $\mu$g) or buffer (control) by oropharyngeal aspiration following isoflurane anesthesia, euthanized 24 h later, and BAL assayed; $n = 5$ mice/time point. Differences between SDF-1 augmentation and control: *, $p = 0.05$; **, $p < 0.02$; NS, not significant. WBC, White blood cell; ABS, absolute number; PMN, polymorphonuclear leukocyte; Mac, macrophage; Lymph, lymphocyte.

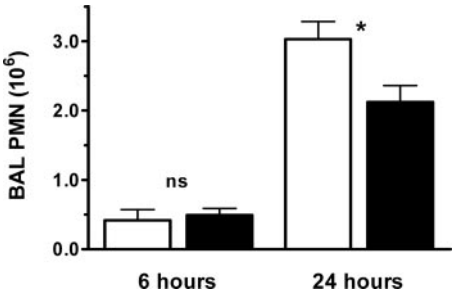


**FIGURE 8.** The effects of SDF-1 blockade on BAL neutrophil content are minimal at 6 h after LPS injury. Mice were treated with LPS and SDF-1 blocking Ab (■) or LPS and control Ab (□) by oropharyngeal aspiration following isoflurane anesthesia and then euthanized and assayed 6 h later; $n = 5–7$ mice/time point. Twenty four-hour blockade data from Fig. 7 is included in the figure for comparison. *, $p = 0.02$; ns, not significant compared with control.

### *Pulmonary SDF-1 blockade predominantly attenuates late neutrophil recruitment to the lung during LPS-induced pneumonitis*

Given the delayed course of SDF-1 induction in the lung following LPS injury, we posited that, unlike the blockade of early cytokines such as KC and MIP-2 (18), SDF-1 blockade would not affect the early recruitment of neutrophils to the lung. We therefore examined a representative early time point (6 h) at which significant airspace neutrophilia is found following LPS exposure (23, 55). As shown in Fig. 8 and Table I, BAL neutrophilia was not altered by SDF-1 blockade at 6 h following LPS exposure (blocked, $0.49 \pm 0.10 \times 10^6$ vs control, $0.42 \pm 0.15 \times 10^6$ cells total). A trend toward lower BAL lymphocyte percentage was seen in the blocked animals, consistent with the previously described role of SDF-1 in lymphocyte recruitment (28, 46). Blood neutrophil levels were unchanged by SDF-1 blockade at this time point (data not shown). These findings further support a role for the CXCR4/SDF-1 axis in neutrophil recruitment during the later phases of ALI.

## Discussion

The recruitment of neutrophils to the lung is a pivotal factor in the development and perpetuation of ALI. A rapid, early influx of mature circulating neutrophils to the injured lung is followed by a slower, sustained recruitment of neutrophils from the bone marrow. Much of the progressive damage to the lung that characterizes ALI is attributable to this second, persistent phase of neutrophilia, and its duration and severity are predictors of mortality (5–7). Although many of the signals responsible for the initial influx of neutrophils to the lung in ALI have been delineated (18), the mechanisms governing the recruitment of this subsequent persistent wave of neutrophils remain unclear.

Hypothetically, cytokines governing the persistent recruitment of neutrophils to the lung in ALI would display two requisite characteristics: delayed and/or persistent expression in lung tissues during the course of ALI and the ability to chemoattract the phenotypically distinct neutrophil population that appears in the circulation due to accelerated marrow release during the progression of lung injury. We report findings that support such a role for the CXCR4/SDF-1 chemokine axis in this process. We examined both human lung samples from patients with ALI and murine lung samples following experimental injury with LPS and found a similar robust pattern of tissue SDF-1 expression during ALI. In mice, both *SDF-1* mRNA and protein are seen to increase in response to lung injury (Fig. 2) in accordance with previous reports examining experimental bleomycin-induced lung fibrosis (34, 36). Furthermore, although IHC (Fig. 1) suggests that SDF-1 expression in the lung may originate from alveolar macrophages and the type II epithelium, further investigation by lung disaggregation and magnetic bead sorting indicate that the major source of SDF-1 release is the lung epithelium, with a much smaller component arising from pulmonary leukocytes (Figs. 3 and 4). Such an anatomic pattern of expression differs from that seen with other CXC cytokines in the lung during ALI in which the alveolar macrophage plays a prominent role (18). Furthermore, dissection of the time course of SDF-1 expression (Fig. 2) suggests that SDF-1 release in ALI is more delayed and sustained by comparison. This temporal pattern of expression is fairly unique among the cytokines released during ALI (23–26) and is compatible with SDF-1 participation in the late recruitment of neutrophils in ALI. Although SDF-1 release by the damaged lung appears to be a response common to a variety of injuries, including allergic (46), fibrotic (34), and ischemic (35), the mechanisms governing SDF-1 expression in the lung in these settings are unclear. Early proinflammatory cytokines such as TNF-$\alpha$ and IL-1$\beta$ have been shown to modulate SDF-1 in both the marrow and skin (56, 57), and an equally important regulator of SDF-1 levels in marrow appears to be its proteolytic degradation by proteases such as matrix metalloproteinase-9 and elastase (31). These mechanisms have yet to be investigated in the injured lung.

Previous reports have suggested that SDF-1 may participate in the recruitment of neutrophils in arthritis (41–44) and peritonitis (45). Interestingly, SDF-1 expression has also been implicated in the trafficking of myeloid progenitors during injury and inflammation to the liver (58, 59) and the peritoneum (60), suggesting that the same chemokine interactions that maintain neutrophils and their precursors within the marrow (29, 38–40) may be effective in recruiting these cells to extramedullary sites. We posited that pulmonary SDF-1 expression during the progression of ALI could similarly serve as a neutrophil chemoattractant. Such a possibility is supported by our chemotaxis studies (Fig. 6). Furthermore, as shown in Fig. 7, selective Ab blockade of pulmonary SDF-1 during LPS pneumonitis significantly attenuates, while SDF-1 augmentation increases, BAL neutrophilia at 24 h, supporting a role for SDF-1 in neutrophil recruitment to the injured lung. Interestingly, we find that an intratracheal SDF-1 blockade does not reduce blood leukocytosis (and may in fact increase it), suggesting that although pulmonary SDF-1 appears to play an important role in the airspace recruitment of neutrophils from blood, lung-derived SDF-1 is unlikely to participate in the release of these cells from the marrow. This may imply that, similar to MIP-2 (61), the actions of pulmonary SDF-1 may be largely compartmentalized to the lung.

In concordance with the temporal pattern of pulmonary SDF-1 expression following injury (Fig. 3), this cytokine appears to be most active in the late recruitment of neutrophils in our experimental model of ALI (Fig. 5). Thus, SDF-1-driven recruitment of neutrophils to the lung appears to be greatest during the period of accelerated neutrophil release from the marrow. One might therefore predict that the population of neutrophils released from the marrow and recruited to the lung during this period would express increased levels of surface CXCR4. Such expression would reflect the relative immaturity of these cells (29, 38–40) and the necessity of SDF-1 for their effective recruitment to the lung. As shown in Fig. 5, in the later period of ALI circulating neutrophils are indeed found to express higher levels of CXCR4 overall (Fig. 5*A*). In addition, $CXCR4^{high}$ cells appear to be selectively recruited to the lungs later in the course of injury (Fig. 5*B*), a finding that is partially reversed with Ab-mediated SDF-1 blockade. The mechanisms underlying the marrow release of $CXCR4^{high}$ neutrophils in this setting are unclear but might include transient internalization

of CXCR4 (39), heterologous desensitization of this receptor (38), or a decrease in marrow SDF-1 through proteolysis or attenuated expression (31, 62). Although reports are conflicting (38, 39), it has also been suggested that CXCR4 may also be increased in senescent cells. It is unlikely, however, that such a mechanism would contribute to our findings because ALI has been shown to retard senescence, particularly in airspace neutrophils (63).

Thus, our data indicate an important role for the CXCR4/SDF-1 axis in the recruitment of neutrophils to the lung during ALI and may suggest one of the mechanisms underlying the later phase of lung inflammation in this condition. It is interesting to note that this cytokine axis has also recently been implicated in the reparative response to lung injury (35) and may therefore represent an overlapping mechanism between inflammatory and reparative responses. Furthermore, given the role of SDF-1 in the homing of metastatic tumor cells (64), our findings that lung inflammation up-regulates SDF-1 may shed light on the previously noted correlation between such inflammation and the promotion of lung metastasis (65, 66).

The persistent inflammatory phase of ALI has been shown to contribute significantly to both the pathogenesis and the subsequent mortality of this disease (5–7). Given its delayed onset and extended course, this period of ALI may be an ideal target for therapeutic intervention. Further investigation of the mechanisms underlying this phase of the disease will be required to address these possibilities.

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## Disclosures

The authors have no financial conflict of interest.

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