ORIGINAL RESEARCH

Platelet-Specific Chemokines Contribute to the Pathogenesis of Acute Lung Injury

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Abstract

Platelets and neutrophils contribute to the development of acute lung injury (ALI). However, the mechanism by which platelets make this contribution is incompletely understood. We investigated whether the two most abundant platelet chemokines, CXCL7, which induces neutrophil chemotaxis and activation, and CXCL4, which does neither, mediate ALI through complementary pathogenic pathways. To examine the role of platelet-derived chemokines in the pathogenesis of ALI using *Cxcl7*$^{-/-}$ and *Cxcl4*$^{-/-}$ knockout mice and mice that express human CXCL7 or CXCL4, we measured levels of chemokines in these mice. ALI was then induced by acid aspiration, and the severity of injury was evaluated by histology and by the presence of neutrophils and protein in the bronchoalveolar lavage fluid. Pulmonary vascular permeability was studied *in vivo* by measuring extravasation of fluorescently labeled dextran. Murine CXCL7, both recombinant and native protein released from platelets, can be N-terminally processed by cathepsin G to yield a biologically active CXCL7 fragment. Although *Cxcl7*$^{-/-}$ mice are protected from lung injury through the preservation of endothelial/epithelial barrier function combined with impaired neutrophils transmigration, *Cxcl4*$^{-/-}$ mice are protected through improved barrier function without affecting neutrophils transmigration to the airways. Sensitivity to ALI is restored by transgenic expression of CXCL7 or CXCL4. Platelet-derived CXCL7 and CXCL4 contribute to the pathogenesis of ALI through complementary effects on neutrophil chemotaxis and through activation and vascular permeability.

Keywords: acute lung injury; platelets; chemokines

Platelets are primary effector cells of hemostasis and thrombosis (1–3). A growing body of evidence suggests that platelets also play a crucial role in the inflammatory response, in part by promoting leukocyte recruitment to sites of vascular injury (4). Recent studies also highlight the unique functions of platelets in the lungs (5) and show that platelets contribute to acute lung injury (ALI) (6).

ALI, which causes significant morbidity and mortality, is a multifactorial syndrome characterized by loss of microvascular barrier function; recruitment, retention, and activation of leukocytes in the lung; and parenchymal injury(7–10). Chemokines that bind to the receptor CXCR2 are important in the recruitment of neutrophils (11). Deletion of the gene encoding CXCR2 in both hematopoietic and nonhematopoietic cells completely blocks neutrophil activation, whereas inhibition of CXCR2 activity attenuates acid-induced ALI by reducing neutrophil recruitment and decreasing

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Clinical Relevance

These studies show a mechanism by which platelets, through the release of platelet-specific chemokines, may influence the degree of acute lung injury and provide important new insights into its pathogenesis. They may also lead to novel therapeutic strategies to modify the severity of this challenging disorder.

vascular permeability within the lung (12). Platelets also contribute to neutrophil-mediated injury by helping to "capture" neutrophils along the lung microvasculature (13). Disruption of platelet-neutrophil aggregates and depletion of platelets reduce neutrophil migration and improve survival in animal models of ALI (14). Activated platelets also promote intravascular thrombosis, which is a common accompaniment of ALI and which contributes to morbidity (15).

Platelets enhance or facilitate leukocyte recruitment primarily through the release of specific chemokines that are stored in α-granules. The most abundant proteins in α-granules are CXCL4 (platelet factor 4) and CXCL7 (platelet basic protein). Mature CXCL4 is a 70-amino acid (aa) protein that promotes thrombosis (16), enhances atherosclerosis (17, 18), inhibits megakaryopoiesis (19), and regulates T-cell and other host immune responses to certain infections (20–22). The contribution of CXCL4 to these processes has been delineated in part through studies of a CXCL4 knockout (*Cxcl4*$^{-/-}$) mouse that has a 1.2-kb deletion that removes all three exons of *Cxcl4* and through using transgenic mice expressing human (h) CXCL4 specifically in platelets (hCXCL4^{+}) (16, 23). At high concentrations at which CXCL4 shows biological activity, it is likely that its affinity for ubiquitous negatively charged cell-surface glycosaminoglycans is more important than its ability to bind specific cell surface receptors (24–27). Although CXCL7 is structurally related to CXCL4, it is cleaved to an N-terminal 70-aa fragment, neutrophil-activating peptide-2 (NAP-2) (CXCL7/NAP-2). CXCL7/NAP-2 has a glutamic acid-leucine-arginine (ELR) motif within its N-terminal sequence that is absent in CXCL4. This sequence is required for binding to CXCR1 and CXCR2, which mediates the migration and activation of neutrophils (2, 28, 29). Human CXCL7/NAP-2 can be produced *in vitro* by N-terminal cleavage of its proform by several serine proteases, including trypsin, chymotrypsin, and cathepsin G (30). *In vivo*, CXCL7 is cleaved primarily by neutrophil cathepsin G (31), which, in turn, regulates neutrophil function (32). Both human and mouse megakaryocytes synthesize CXCL7. The proteins differ in size (94 and 79 aa, respectively), but mouse (m) CXCL7 can also be N-terminally cleaved to mCXCL7/NAP-2 (33).

As mentioned, CXCR2 is an important receptor that has been implicated in pathophysiologic events associated with ALI. In mice, CXCR2 mediates the chemotactic activity of CXCL1 (KC), CXCL2 (MIP-2), CXCL5 (LIX), and CXCL15 (lungkine). These chemokines are important for neutrophil recruitment into rodents' lungs (9, 34). CXCR2 also binds CXCL7, and both have been reported recently to promote leukocyte migration through thrombi (35).

On the basis of their abundance in platelets, CXCL7 and CXCL4 are likely to be the most abundant proteins released at sites of inflammation. However, the specific role of platelet-derived CXCL7/NAP-2 in the development of ALI and the contribution of CXCL4 with its more pleiotropic effects and lack of known receptor in the lung have not been well defined. The specific roles of individual chemokines have been difficult to delineate because they are all released simultaneously in wild-type (WT) animals. To address the specific contribution of CXCL7/NAP-2 and CXCL4 to the development of ALI, we used transgenic mice either lacking these chemokines individually or expressing their human counterparts individually on knockout backgrounds. Our studies show that both CXCL7/NAP-2 and CXCL4 contribute to the development of ALI through complementary effects on neutrophil transmigration and pulmonary vascular permeability.

Materials and Methods

Reagents

All reagents, unless specified otherwise, were from Sigma (St. Louis, MO).

Expression of Murine Platelet-Specific Chemokines

Recombinant proteins were expressed by subcloning complementary DNA into a pT7-7 prokaryote expression vector and expressing proteins in BL21DE3 pLysS bacteria after isopropyl β-D-1-thiogalactopyranoside induction, as described by our group (36). Proteins were purified on heparin agarose followed by liquid chromatography. Both full-length mCXCL7 and mCXCL7/NAP-2 proteins react with rabbit polyclonal antimouse CXCL7 antibody produced against peptides specific for mCXCL7. Their endotoxin levels were <0.005 ng/μg as determined by the limulus amebocyte lysate assay. The human and mouse CXCL7/NAP-2 used for chemotaxis of murine neutrophils were from Peprotech (Rocky Hill, NJ) and BioLegend (San Diego, CA), respectively.

Neutrophil Isolation and Chemotaxis

After approval from the institutional review board of Childrens' Hospital of Philadelphia and after informed written consent, we obtained human blood samples from healthy adult donors via venipuncture. Human neutrophils were isolated by centrifugation in Lympholyte Polyseparation Medium (Cedarlane Laboratories, Burlington, NC) according to the manufacturer's instructions, labeled with Calcein-AM (1 μM final concentration), washed, counted, and diluted to a final concentration of 3×10^6 cells/ml in phosphate-buffered saline (DPBS; Life Technologies, Grand Island, NY). Chemotaxis of human neutrophils was assessed using the Neuro Probe ChemoTx Disposable Chemotaxis system (Neuro Probe, Gaithersburg, MD) with the Victor Plate Reader (Perkin Elmer, Waltham, MA). *N*-formyl-methionyl-leucyl-phenylalanine (37) was used as positive control.

Mouse neutrophils were isolated from the bone marrow of WT mice (38) and purified using the Neutrophil Isolation Kit, (Miltenyi Biotec, Bergisch Gladbach, Germany). Chemotaxis assays were performed using 1×10^6 cells/ml of Calcein-labeled cells using Transwell unit (3 μm, Corning Costar, Corning, NY) (39).

Cxcl7$^{-/-}$ and *Cxcl4*$^{-/-}$ Mice and hCXCL7^{+} and hCXCL4^{+} Transgenic Mice

We generated *Cxcl7*$^{-/-}$ and *Cxcl4*$^{-/-}$ mice by deleting all three exons of the respective

genes using a targeted homologous recombination strategy (16, 40). Heterozygous mice were initially bred together to obtain WT and knockout mice. The hCXCL7^{+} and hCXCL4^{+} transgenic mice express the respective protein in a platelet-specific fashion (23). Mice denoted as hCXCL7^{+}/*Cxcl7*$^{-/-}$, hCXCL4^{+}/*Cxcl7*$^{-/-}$, and hCXCL4^{+}/*Cxcl4*$^{-/-}$ were hemizygous for the transgene and were studied in parallel with their respective knockout littermates. All lines were backcrossed >10 generations onto a C57BL/6J (Jackson Laboratories, Bar Harbor, ME) background before study.

Acid Lung Injury and Immunostaining of Lung Tissue

Mice (6–8 weeks old, 20–25 g) were studied after approval by the Institutional Animal Care and Use Committee of Children's Hospital of Philadelphia. Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine. Tracheae and jugular veins were surgically exposed. A 1% solution of fluorescein isothiocyanate (FITC-dextran, 40 kD; Sigma) in DPBS was injected into the jugular vein. Acid aspiration was induced by intratracheal injection of 0.1 N HCl. Four hours later, lung injury, vascular permeability, and extravasation of neutrophils into lung parenchyma and airways were evaluated as described (41). In some experiments, sections (5 μm) cut from formalin-fixed and paraffin-embedded lung tissues were immunostained using rabbit anti-mCXCL7 (Bethyl Laboratories, Montgomery, TX) or normal rabbit sera, followed by a biotinylated-antirabbit antibody (Vector Laboratories, Burlingame, CA). (For additional methodological details, *see* online supplement.)

Results

mCXCL7 Is Processed to Yield Biologically Active mCXCL7/NAP-2

Proteolytic cleavage of hCXCL7 results in a mature protein (NAP-2) with an exposed ELR N-terminus (32) that interacts with chemokine receptors CXCR1 and CXCR2 to induce neutrophil migration (28, 42). Murine full-length CXCL7 has only ~50% identity with its human counterpart (*see* Figure E1 in the online supplement). To determine whether murine and human CXCL7 undergo similar post-translational processing and thereby evolve similar biological activities, we expressed full-length mCXCL7 and examined whether it, too, can be proteolyzed to yield a biologically active mCXCL7/NAP-2 fragment capable of binding to CXCR2. We examined N-terminal cleavage of full-length recombinant mCXCL7 after digestion by cathepsin G. Bands corresponding in size to recombinant CXCL7/NAP-2 that were reactive with antimouse CXCL7 antibody were identified (Figure 1A) after digestion of recombinant mCXCL7 and in platelet lysates from WT mice, but the band was absent as expected in platelet lysates from *Cxcl7*$^{-/-}$ mice (Figure 1A and Figure E2). These data show that mouse CXCL7, like its human counterpart (43), can be cleaved by cathepsin G to release an N-terminal ELR CXC chemokine NAP-2. Next, we investigated whether purified recombinant mCXCL7/NAP-2, like its human counterpart (hCXCL7/NAP-2), induces chemotaxis of isolated human neutrophils (Figure 1B). We also show here that mCXCL7/NAP-2, but not full-length mCXCL7, supported human neutrophil chemotaxis (Figure 1B). Both mCXCL7/NAP-2 and hCXCL7/NAP-2 supported human neutrophil chemotaxis in a concentration-dependent manner (Figure E3). Both hCXCL7/NAP-2 and mCXCL7/NAP-2 also supported the migration of murine neutrophils (Figure 1C). This finding is consistent with the previously identified role of CXCL7/NAP-2 in a mouse model of leukocyte migration through thrombi (35) and is relevant to our findings in mice overexpressing hCXCL7.

Characterization of the Murine Lines

Mice from all knockout and human CXC-expressing lines were born at the expected Mendelian ratios. None of the mice displayed an overt phenotype (16) (data not shown). Western blot analysis of platelet lysates from *Cxcl7*$^{-/-}$ and *Cxcl4*$^{-/-}$ mice (Figure 1A, *right panel*; Figure E2) (16), as well as specific ELISAs of their sera (*see* Table E1 in the online supplement), confirmed the absence of the respective proteins when compared with samples from WT mice. *Cxcl4*$^{-/-}$ mice have near-normal levels of mCXCL7 protein, whereas *Cxcl7*$^{-/-}$ mice express ~25% of the normal levels of mCXCL4 (Table E1). The genes for these CXC chemokines are ~4 kb apart in both mice and humans (44) (Figure E4). Therefore, we measured the effects of these deletions on the expression of other chemokines. Serum levels of mCXCL5 in *Cxcl4*$^{-/-}$ mice were ~ 40% of those in WT mice, whereas mCXCL5 was almost absent from *Cxcl7*$^{-/-}$ sera (Table E1). The levels of the other linked chemokine, CXCL1, were normal in all the animals studied. We infer that the deletions of the *CXCL7*$^{-/-}$ and *CXCL4*$^{-/-}$ genes affect the regulatory organization around the *Cxcl5* gene, decreasing its expression.

ALI in Mice Lacking CXCL7 or CXCL4

We used acid inhalation as our model of lung injury. Acid or saline as a control was injected intratracheally into WT, *Cxcl7*$^{-/-}$, and *Cxcl4*$^{-/-}$ mice. The chest was then opened and the intact lungs were inspected visually. Representative pictures of whole lungs harvested 4 hours after injury and saline control are shown as the insert (Figure 2A). The damage was scored on a scale of 1 (no injury) to 10 (severe injury), as described (45), and was validated by us previously (41). On the basis of ALI scores, injury to the lungs of saline-injected WT mice (Figure 2A, *white bar* and *saline dotted line*) was low, in stark contrast to the damage seen in the lungs of acid-infused WT mice (Figure 2A, *black bars*). Lung injury in *Cxcl7*$^{-/-}$ or *Cxcl4*$^{-/-}$ mice exposed to acid was significantly less severe (Figure 2A, *gray bars*) than in WT mice.

In parallel experiments, lungs from animals 4 hours after injection of saline or HCl were inflated, fixed with formalin and paraffin, sectioned, and stained using hematoxylin and eosin. Histological inspection of sections from the saline-control and acid-injured lungs taken from WT, *Cxcl7*$^{-/-}$, and *Cxcl4*$^{-/-}$ mice (Figure 2B) affirmed the results of visual inspection. After acid aspiration, lungs from mice lacking CXCL7 showed essentially normal parenchymal architecture, minimal interstitial edema, and fewer leukocytes and red blood cells in alveolar spaces compared with lungs from HCl-injured WT mice.

Deletion of Cxcl7 or Cxcl4 Decreases Pulmonary Vascular Permeability

To characterize pulmonary vascular permeability after acid injury, we first measured total protein in bronchoalveolar lavage fluid (BALF). Protein content in BALF from acid-injured WT mice was approximately threefold higher than in *Cxcl7*$^{-/-}$ or *Cxcl4*$^{-/-}$ mice (Figure 3A,

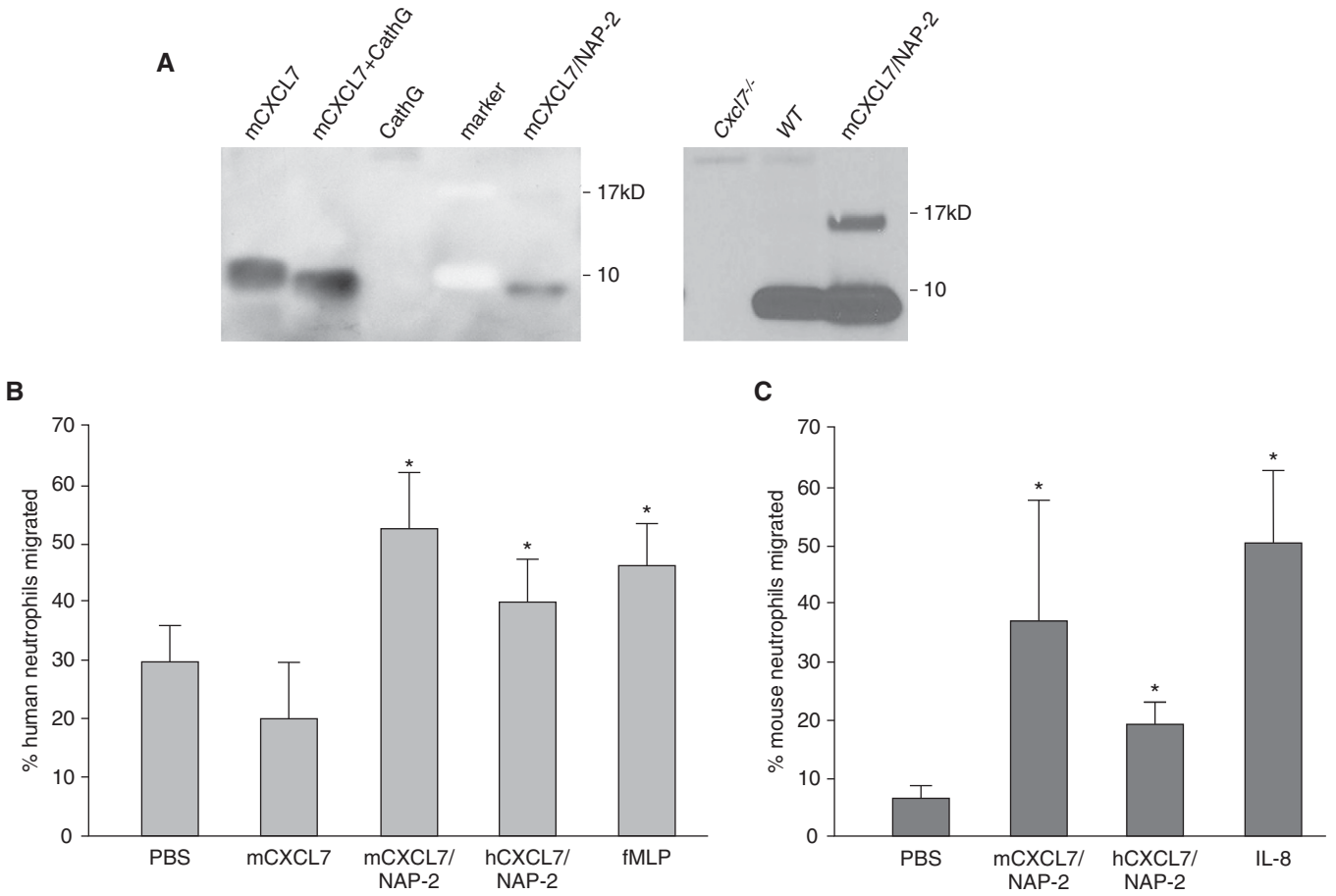

Figure 1. mCXCL7 can be processed to yield biologically active mCXCL7/NAP-2. (*A*) Western blots using anti-mCXCL7 antibody. *Left*: Purified recombinant mCXCL7 (*lane 1*) was digested with CathG (*lane 2*). After digestion, bands corresponding in size to recombinant mCXCL7/NAP-2 are seen. *Right*: Lysates of platelets isolated from *Cxcl7*$^{-/-}$ and WT mice. (*B*) Chemotaxis of human neutrophils (NE) toward mCXCL7 and hCXCL7/NAP-2 (50 nM) compared with control buffer or fMLP (1 μM). (*C*) Chemotaxis of mouse NE toward hCXCL7/NAP-2 and mCXCL7/NAP-2 (15 nM) compared with control buffer (PBS) or IL-8 (5 nM). Results for chemotaxis (% of NE migrated) are from two to three experiments performed on different human donors or animals in duplicate (mean ± SD). *$P < 0.05$ versus PBS. CathG, cathepsin G; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; hCXCL7, human recombinant CXCL7; mCXCL7, mouse CXCL7; NAP-2, neutrophil-activating peptide-2; WT, wild type.

black and *gray bars*). Protein content in BALF from acid-injured *Cxcl7*$^{-/-}$ and *Cxcl4*$^{-/-}$ mice was comparable to the levels in saline-control animals (Figure 3A, *white bar* and *NaCl dotted line*). These results suggest that lung injury was associated with compromised capillary-epithelial barrier function and increased vascular permeability in WT mice, and that *Cxcl7*$^{-/-}$ and *Cxcl4*$^{-/-}$ mice were protected from acid-induced injury. To dissociate systemic (blood or plasma) protein influx into the airways from a local increase in total protein caused by injured airways, we assessed vascular permeability after intravenous injection of FITC-dextran. Diffusion of FITC-dextran from the circulation into BALF from WT, *Cxcl7*$^{-/-}$, and *Cxcl4*$^{-/-}$ mice was measured after acid aspiration, as described previously (41). Little FITC-dextran was detected in the BALF of WT mice injected with saline (Figure 3B, *NaCl dotted line*). Levels increased approximately fivefold after acid aspiration in WT mice, but only increased approximately twofold in *Cxcl7*$^{-/-}$ mice (Figure 3B, *black* and *light gray bars*). Similarly, FITC-dextran in BALF from acid-injured *Cxcl4*$^{-/-}$ mice was only slightly increased compared with that in BALF from control mice injected with saline (Figure 3B, *dark gray bar* and *NaCl dotted line*), but it was decreased significantly compared with FITC-dextran in BALF from WT mice.

The increase in lung permeability in WT mice was accompanied by increased neutrophil transmigration into the airways compared with mice injected with saline, as anticipated (Figure 3C, *black* and *white bars*). BALF from WT mice also contained significantly more neutrophils after acid injury than did BALF from injured *Cxcl7*$^{-/-}$ mice (Figure 3C, *black* and *light gray bars*, respectively). Surprisingly and in contrast to that in *Cxcl7*$^{-/-}$ mice, BALF from acid-injured *Cxcl4*$^{-/-}$ mice contained only slightly fewer neutrophils than did BALF from acid-injured WT mice (Figure 3C, *dark gray bars*).

Lung Injury in Mice Overexpressing Human CXCL7 or CXCL4

Because *Cxcl7*$^{-/-}$ mice expressed only one-fourth the mCXCL4 found in WT mice and no detectable mCXCL5 (Table E1),

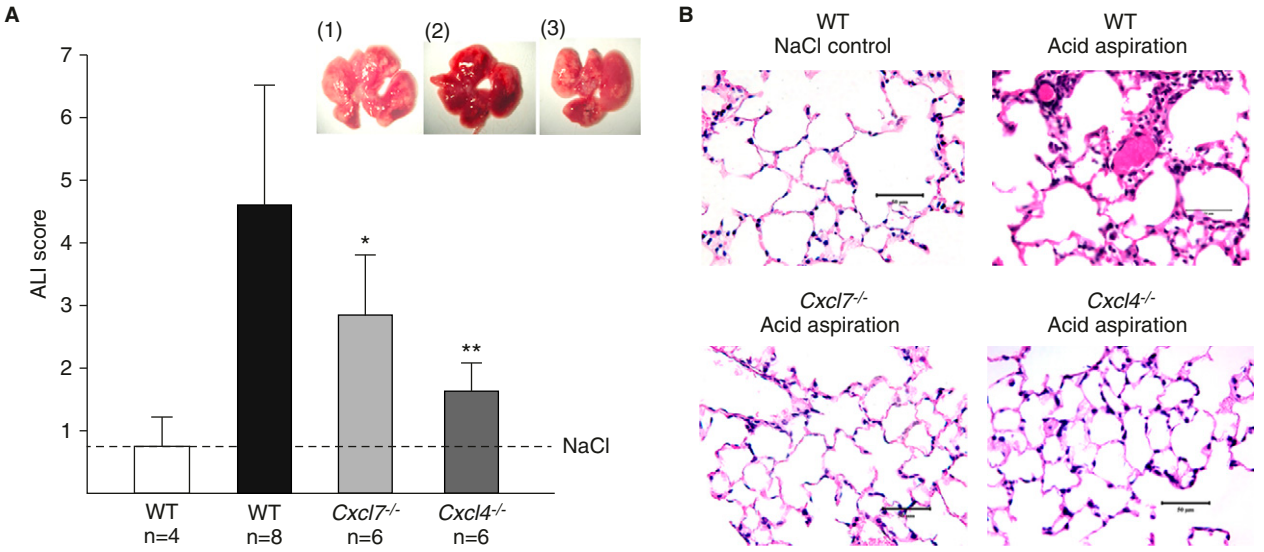

Figure 2. Lung injury in *Cxcl7*$^{-/-}$ and *Cxcl4*$^{-/-}$ mice. (*A*) Average acute lung injury (ALI) score was measured in four WT mice that were injected intratracheally with NaCl (*white bars* and *dotted line*) and in six to eight mice that were injected intratracheally with HCl in each group, as indicated. *$P <$ 0.05 and **$P < 0.01$ as compared with WT mice after acid injury. *Inset*: Representative images of freshly noninflated whole lungs after injection of (*1*) 0.9% NaCl in WT, (*2*) 0.1N HCl in WT, and (*3*) 0.1N HCl in *Cxcl7*$^{-/-}$ mice. (*B*) Hemotoxylin and eosin staining of lung sections collected 4 hours after intratracheal injection of either 0.9% NaCl (control) in WT mice or 0.1N HCl (acid aspiration) in WT, *Cxcl7*$^{-/-}$, and *Cxcl4*$^{-/-}$ mice. Representative lung sections (original magnification × 400; *scale bars*: 50 μm).

we next examined whether overexpressing human CXCL7 or CXCL4 on a *Cxcl7*$^{-/-}$ or *Cxcl4*$^{-/-}$ background would restore the severity of lung injury. We studied hCXCL7^{+}/*Cxcl7*$^{-/-}$ and hCXCL4^{+}/Cxcl7$^{-/-}$ mice that express human CXCL7 and CXCL4, respectively, and have decreased mCXCL4 and no mCXCL5 or mCXCL7. The hCXCL4 expression transgene (23) was also crossed onto the same *Cxcl4*$^{-/-}$ genetic background (hCXCL4^{+}/*Cxcl4*$^{-/-}$).

We scored the injury to intact lungs visually after acid aspiration (41). In contrast to the outcomes in *Cxcl7*$^{-/-}$ and *Cxcl4*$^{-/-}$ mice (Figure 2A), ALI scores in hCXCL7^{+}/*Cxcl7*$^{-/-}$, hCXCL4^{+}/*Cxcl4*$^{-/-}$, and hCXCL4^{+}/*Cxcl7*$^{-/-}$ mice were comparable to each other and slightly higher than those in injured WT mice, although the difference did not reach statistical significance (Figure 4A). This indicates that introducing human CXCL7 or CXCL4 rescued the knockout phenotypes. These findings were confirmed by histological analysis of hematoxylin and eosin–stained lung sections (Figure 4B). Lung sections from mice overexpressing human CXCL7 or CXCL4 after acid aspiration showed increased

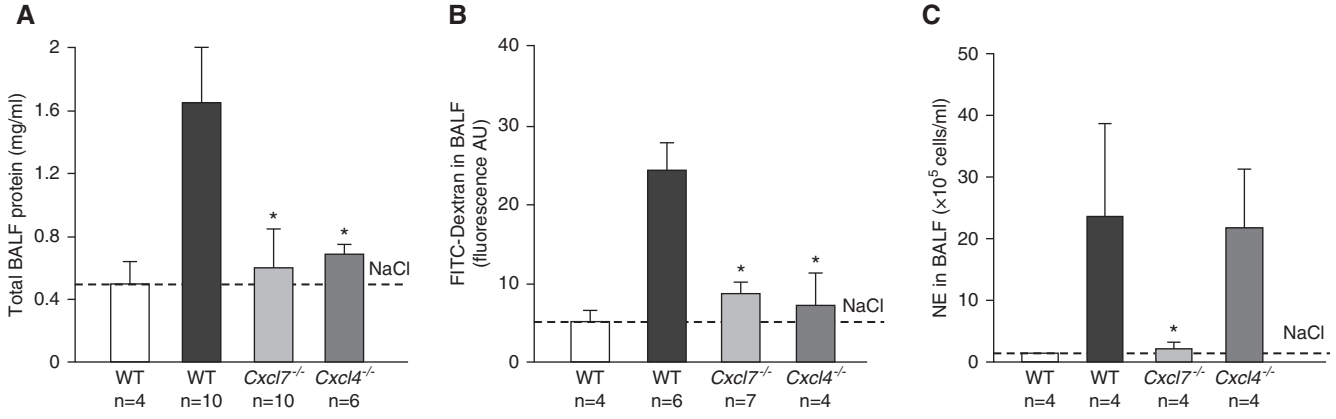

Figure 3. Vascular permeability in *Cxcl7*$^{-/-}$ and *Cxcl4*$^{-/-}$ mice. (*A*) Total protein in bronchoalveolar lavage fluid (BALF) from *Cxcl7*$^{-/-}$ and *Cxcl4*$^{-/-}$ mice was compared with that from WT animals after acid injury and with mice injected with NaCl (*dotted line*). BALF was collected 4 hours after intratracheal injection of 0.1N HCl or 0.9% NaCl. Mean ± SD of 4–10 animals per group are shown (*$P < 0.01$ versus WT). (*B*) Permeability was also measured by fluorescein isothiocyanate (FITC-dextran) content. Mean ± SD of four to seven animals per group are shown (*$P < 0.01$ versus WT). (*C*) Number of NE in BALF from acid-injured lungs of WT, *Cxcl4*$^{-/-}$, and *Cxcl7*$^{-/-}$ mice. Mean ± SD from four mice in each experimental group are shown (*$P < 0.01$ versus WT). fluorescence AU, fluorescence arbitrary units.

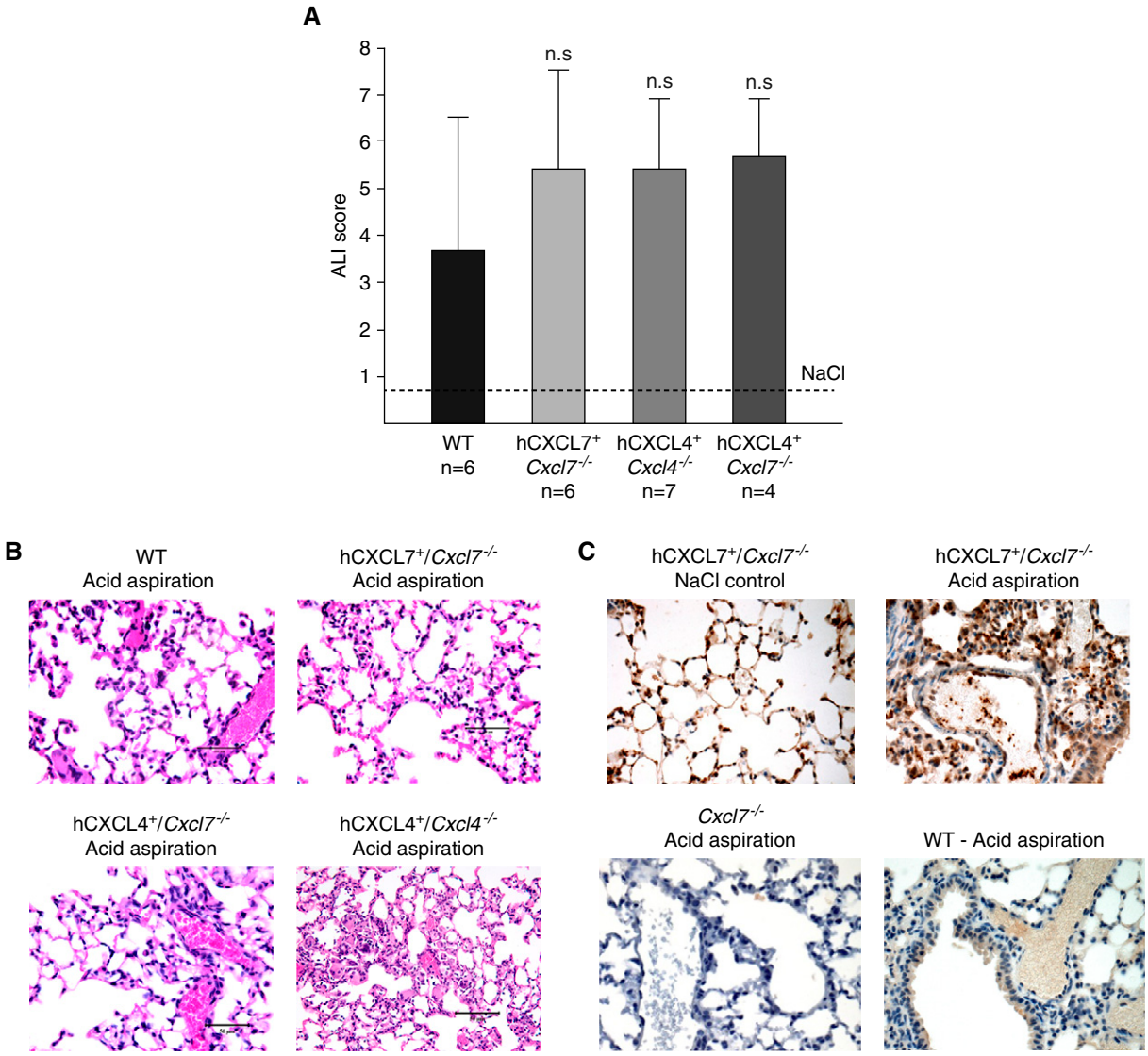

Figure 4. Lung injury in $Cxcl7^{-/-}$ and $Cxcl4^{-/-}$ mice expressing either hCXCL7 or hCXCL4. (*A*) BALF was collected 4 hours after intratracheal injection of 0.1N HCl or 0.9% NaCl. Average ALI score was measured in mice injected with NaCl (*dotted line*) and with HCl in four to seven mice in each group as indicated and compared with that of WT mice after acid injury. (*B*) Hemotoxylin and eosin staining of lung sections collected 4 hours after acid aspiration in WT, hCXCL7^{+}/$Cxcl7^{-/-}$, hCXCL7^{+}/$Cxcl7^{-/-}$, and hCXCL7^{+}/$Cxcl7^{-/-}$ mice. Representative lung sections (original magnification × 400; *scale bars*: 50 μm). (*C*) Experiments were performed as in (*B*), but lung sections were stained with rabbit antibody against hCXCL7, followed by antirabbit secondary antibody. In both (*B*) and (*C*), images are representative of results from three to four mice in each treatment group. n.s., not significant; hCXCL4, human CXCL4.

alveolar septal thickening and congestion, as was seen in WT mice. To assess the presence of platelets and the release of hCXC7, lung sections from control and acid-injured hCXC7^{+}/$Cxcl7^{-/-}$, $Cxcl7^{-/-}$, and WT mice after acid aspiration were stained with anti-human CXCL7 antibody (Figure 4C). hCXCL7 was found both in association with and apart from platelets in injured hCXC7^{+}/$Cxcl7^{-/-}$ lungs (Figure 4C, *top right panel*), compared with minimal staining in control mice injected with saline (Figure 4C, *top left panel*). hCXCL7 was also evident in the epithelial airways of injured hCXC7^{+}/$Cxcl7^{-/-}$ lungs. This suggests that CXCL7 released from activated platelets might bind to the epithelium. As expected, no staining for hCXCL7 was seen in injured $Cxcl7^{-/-}$ and WT mice because the anti-human CXCL7 antibody used does not cross-react with murine CXCl7. (Figure 4C, *top left* and *right panels*, respectively).

We then measured total protein in BALF and permeability to FITC-dextran (Figures 5A and B, respectively) in these transgenic mice. In agreement with the ALI scores (Figure 4A), and in contrast to results in knockout mice (Figures 3A and 3B), the protein and FITC-dextran levels in BALF of injured hCXCL7^{+}/$Cxcl7^{-/-}$, hCXCL4^{+}/$Cxcl7^{-/-}$, and hCXCL4^{+}/$Cxcl4^{-/-}$ mice were comparable to those seen in injured WT mice (Figures 5A and 5B). The lower

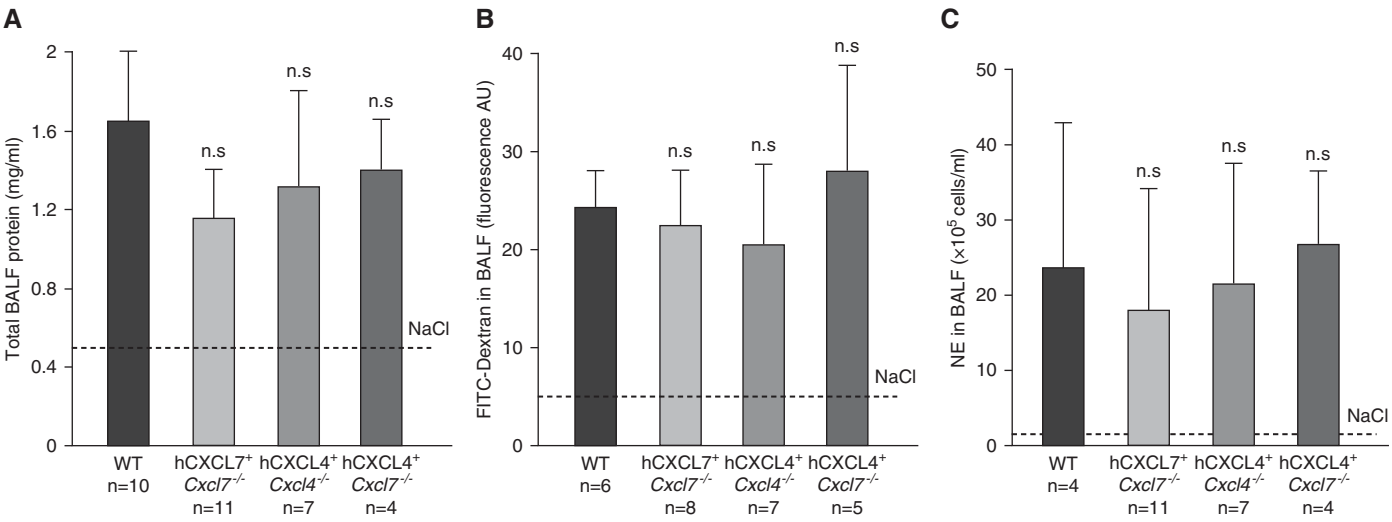

Figure 5. Vascular permeability in *Cxcl7*$^{-/-}$ and *Cxcl4*$^{-/-}$ mice overexpressing either hCXCL7 or hCXCL4. (*A*) Total protein in BALF from hCXCL7^{+}/*Cxcl7*$^{-/-}$, hCXCL4^{+}/*Cxcl7*$^{-/-}$, or hCXCL4^{+}/*Cxcl4*$^{-/-}$ mice was compared with that from WT animals after acid injury. *Dotted line*: intratracheal injection of 0.9% NaCl (no injury). Mean ± SD are shown for 4–11 mice. (*B*) Permeability was measured in WT, hCXCL7^{+}/*Cxcl7*$^{-/-}$, hCXCL4^{+}/*Cxcl7*$^{-/-}$, and hCXCL4^{+}/*Cxcl4*$^{-/-}$ mice by FITC-dextran content in BALF collected 4 hours after intratracheal injection of 0.1N HCl or 0.9% NaCl (*dotted line*). Mean ± SD are shown for five to seven mice. (*C*) Number of NE in BALF from acid-injured lungs of WT, hCXCL7^{+}/*Cxcl7*$^{-/-}$, hCXCL4^{+}/*Cxcl7*$^{-/-}$, and hCXCL4^{+}/*Cxcl4*$^{-/-}$ mice. Mean ± SD from four mice in each experimental group are shown. n.s. as compared with WT mice.

levels of protein in BALF from hCXCL7^{+}/*Cxcl7*$^{-/-}$ mice, compared with those in BALF from WT and hCXCL4^{+}/*Cxcl4*$^{-/-}$ mice, did not reach statistical significance (Figures 5A and 5B, *light* and *dark gray* and *black bars*). The increase in lung permeability in injured overexpressing mice was associated with an increased efflux of neutrophils into lung parenchyma and airways after injury, reaching levels comparable to those seen in injured WT mice (Figure 5C). This indicates that restoring hCXCL7 or hCXCL4 expression alone in the respective knockout mouse background increases its response to acid injury.

We performed additional investigations to try to understand the apparent differences between the number of neutrophils in the pulmonary airways and the severity of injury as judged by protein content in airways of *Cxcl4*$^{-/-}$ mice compared with *Cxcl7*$^{-/-}$ mice that express mCXCL4 (Table E1). Because *Cxcl4*$^{-/-}$ mice express CXCL7, which we have shown is activated to chemoattract neutrophils *in vitro* and *in vivo*, we studied the effect of restoring hCXCL4 or hCXCL7 on the clean *Cxcl7*$^{-/-}$ background. To do so, we immunostained lung sections after acid aspiration to identify intravascular or interstitial neutrophils (Figure E5A). Restoring either hCXCL7 or hCXCL4 on the CxCl7$^{-/-}$ background increased the number of neutrophils in injured areas (Figure E5B). CXCL4, unlike CXCL7, has not been shown to attract neutrophils (28). This led us to investigate the direct effects of CXCL4 on endothelial barrier function as a potential mechanism by which it contributes to ALI. To do so, we first investigated whether the vascular low-density lipoprotein–related receptor (LRP), a receptor that has been shown to bind CXCL4 (46) and has been implicated in pulmonary vascular permeability (41), contributes to ALI in CXCL4-overexpressing mice. To do so, we pretreated mice with the LRP antagonist Fc-RAP or an Fc control 5 minutes before acid aspiration. CXCL4-overexpressing mice pretreated with the LRP inhibitor Fc-RAP, but not an Fc control, showed an ~50% reduction in pulmonary vascular permeability on the basis of total protein and FITC-dextran content in BALF after injury (Figure E6). This outcome suggests a direct effect of CXCL4 on endothelial permeability. Moreover, the permeability of endothelial cell monolayers is increased by rhCXCL4 as well as in the presence of hCXCL7 (Figure E7).

Discussion

Migration of neutrophils in response to the N-terminal ELR subfamily of CXC chemokines is an important step in the pathogenesis of ALI (47). The murine model of acid-induced lung injury we used has helped elucidate the contribution of CXC chemokines to induced neutrophil migration (7). The neutrophil content of lungs and BALF correlates with the severity of ALI, and the severity of injury can be mitigated by neutrophil depletion as well as by a deficiency of CXCR2, a receptor that binds ELR-CXC chemokines and contributes to the chemoattraction and activation of neutrophils (11, 12).

The severity of ALI is also attenuated by depletion or inhibition of platelets in this model (14). We hypothesized that activated platelets that traverse the endothelial/epithelial barrier and megakaryocytes entrapped in the lungs contribute to the development of ALI through local release of their large stores of CXCL7 and CXCL4 (1), which recruit neutrophils and may, in turn, further impair barrier function. The data presented in this paper are consistent with this hypothesis and demonstrate that both CXCL7 and CXCL4 contribute to the pathogenesis of lung injury in the acid aspiration mouse model of ALI. ALI was less severe in mice lacking either CXCL7 (*Cxcl7*$^{-/-}$) or CXCL4 (*Cxcl4*$^{-/-}$) compared with WT mice. Because *Cxcl7*$^{-/-}$ mice expressed only one-fourth the mCXCL4

found in WT mice and no detectable mCXCL5, we studied hCXCL7^{+}/*Cxcl7*$^{-/-}$ and hCXCL4^{+}/Cxcl7$^{-/-}$ mice that express human CXCL7 and CXCL4, respectively, and have decreased mCXCL4 and no mCXCL5 or mCXCL7. The expression of either human CXCL7 or CXCL4 on these knockout backgrounds restored the severity of lung injury to that seen in concurrently studied WT control mice. Although we observed a trend suggesting more severe injury in mice overexpressing hCXCL7 or CXCL4 compared with WT mice, the differences did not reach statistical significance.

Limitations in our methodology and conclusions must be noted. The aspiration model is limited by the heterogeneous delivery of acid, and visual inspection, histologic sampling, and global assessment of vascular permeability might not reflect important regional variations that would be evident using computed tomography accompanied by measures of subsegmental volumes and regional gas exchange. Nevertheless, taken together, our macroscopic, histological, and vascular permeability data indicate that restoring hCXCL7 or hCXCL4 expression alone in the respective knockout background restores the response to acid injury and supports the conclusion that CXCL7 and CXCL4 each individually contribute to the altered phenotype in acid-injured knockout mice.

CXCL7 and CXCL4 are synthesized almost exclusively in megakaryocytes, stored in platelet α-granules, and released from these granules when platelets are activated. In humans, it is estimated that micromolar concentrations of both chemokines are found at sites of platelet activation (1). Our studies indicate that mouse CXCL7, like its human counterpart, can be cleaved by cathepsin G to release an N-terminal ELR CXC chemokine NAP-2, which attracts neutrophils. This finding is consistent with the previously identified role of NAP-2 in a mouse model of leukocyte migration through thrombi (35). Therefore, one mechanism by which platelet CXCL7 may contribute to ALI is through the release by neutrophils of cathepsin G, which cleaves the full-length protein, exposing an N-terminal ELR sequence that attracts and activates additional neutrophils at sites of inflammation.

Understanding how CXCL4 contributes to ALI is more complex. CXCL4 does not have an N-terminal ELR sequence (28). Consequently, CXCL4 does not bind to CXCR2 in humans or mice and has not been shown to activate neutrophils or to directly induce their chemotaxis (28, 43). It remains unclear how the platelet-specific chemokine CXCL4 indirectly leads to a similar pathology. In a murine model of LPS-induced ALI, platelet depletion abrogated lung neutrophil infiltration (48), and antibodies to the platelet-derived chemokines CCL5 and CXCL4 markedly diminished neutrophil efflux and lung permeability (49). These observations are consistent with our phenotypic findings that gene deletion of CXCL4 is protective and that overexpression of CXCL4 restores the WT phenotype after acid injury. Biologically active heterodimers of CCL5-CXCL4 form in ALI, and disruption of these complexes abolishes lung edema and neutrophil infiltration (49). However, in our model, CXCL4 did not exacerbate lung injury by directly enhancing neutrophil efflux into the airways. Additional studies are needed to determine if the extent of chemokine depletion in the lung by antibody or blocking peptides versus gene deletion, severity of injury, and diverse readouts contribute to these differences in outcome.

Cxcl4$^{-/-}$ mice express CXCL7, CXCL1, CXCL5, and other cytokines that might stimulate neutrophil transmigration and activation. On the other hand, loss of pulmonary vascular permeability and recruitment of neutrophils into the airways in mice overexpressing CXCL4 on both the *Cxcl4*$^{-/-}$ and the *Cxcl7*$^{-/-}$ backgrounds suggest that CXCL4 does not contribute to ALI in this model by directly enhancing neutrophil migration.

The mechanism by which CXCL4 contributes to compromised pulmonary/capillary barrier function is unclear. CXCL4 signals through vascular LRP (46, 50), which enhances vascular permeability when engaged by small cationic peptides (41), and similar effects of LRP signaling have been implicated in the regulation of blood-brain barrier function (51). CXCL4-mediated enhancement of vascular permeability may facilitate the egress of neutrophils into the lung parenchyma and BALF in response to injury, leading to the release of high local concentrations of neutrophil-stimulating chemokines and other mediators of inflammation and tissue injury. In support of this proposed mechanism, pretreatment with an LRP inhibitor reduced pulmonary vascular permeability in the CXCL4-overexpressing mice. Thus, because LRP has been shown to play a major role in barrier function and because it has been shown that CXCL4 binds and signals through LRP, we strongly suggest that LRP is the pulmonary endothelial receptor that mediates CXCL4 effects on vascular permeability. However, because RAP is a general antagonist for several receptors from the low-density lipoprotein–like superfamily, we cannot exclude the contribution of other receptors such as low-density lipoprotein and very low-density lipoprotein either directly or indirectly, secondary to inhibition of those receptors by systemic injection of Fc-RAP.

Other mechanisms are possible and require further study. CXCL4 has a strongly cationic surface with a high affinity for repetitive negatively charged molecules (25, 52). CXCL4 binds to DNA (preliminary observations) exposed at sites of inflammation in the form of neutrophil extravascular traps. Our preliminary studies show that CXCL4 displaces histones from the DNA within neutrophil extravascular traps, which may injure the pulmonary vasculature and the adjacent epithelial barrier (53, 54). Alternatively, binding of CXCL4 to cell-surface glycosaminoglycans (55) might compete with CXCL7, CXCL5, and other CXC chemokines for binding to Duffy antigen/receptor for chemokines (56), thereby impairing their removal from sites of inflammation (22). This model would be consistent with the reported role of CXCL5 in ALI (34) and would help explain how overexpression of CXCL4 can substitute for the absence of CXCL7, notwithstanding the differences in their effects on attracting and activating neutrophils to the injured lung. Clearly, additional studies will be needed to delineate among these and other possible mechanisms to account for the contribution of CXCL4 to lung injury.

Conclusions

In summary, our studies demonstrate that the two most abundant and platelet-predominant chemokines, CXCL7 and CXCL4, contribute to the development of ALI. Full-length murine CXCL7 can be processed by cathepsin G to N-terminally truncated CXCL7/NAP-2, which directly activates neutrophils. Unexpectedly, CXCL4, which cannot bind to CXCR2 and is not known to be a potent attractant or

activator of neutrophils, also exacerbates ALI, and in this model, it does so independently of CXCL7. The effect of CXCL4 appears to be mediated primarily through a complementary impairment of pulmonary capillary permeability permitting egress of neutrophils into inflamed tissue. Additional studies are needed to delineate the mechanism of action of CXCL4 and to determine if inhibiting the cytokine or its "receptors" might play a role in mitigating ALI in the clinical setting. ■

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