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*J Immunol* 2004; 172:3860-3868; doi: 10.4049/jimmunol.172.6.3860

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CXCR2 Is Critical to Hyperoxia-Induced Lung Injury

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Hyperoxia-induced lung injury is characterized by infiltration of activated neutrophils in conjunction with endothelial and epithelial cell injury, followed by fibrogenesis. Specific mechanisms recruiting neutrophils to the lung during hyperoxia-induced lung injury have not been fully elucidated. Because CXCL1 and CXCL2/3, acting through CXCR2, are potent neutrophil chemoattractants, we investigated their role in mediating hyperoxia-induced lung injury. Under variable concentrations of oxygen, murine survival during hyperoxia-induced lung injury was dose dependent. Eighty percent oxygen was associated with 50% mortality at 6 days, while greater oxygen concentrations were more lethal. Using 80% oxygen, we found that lungs harvested at day 6 demonstrated markedly increased neutrophil sequestration and lung injury. Expression of CXCR2 ligands paralleled neutrophil recruitment to the lung and CXCR2 mRNA expression. Inhibition of CXC chemokine ligands/CXCR2 interaction using CXCR2+/− mice exposed to hyperoxia significantly reduced neutrophil sequestration and lung injury, and led to a significant survival advantage as compared with CXCR2+/+ mice. These findings demonstrate that CXC chemokine ligand/CXCR2 biological axis is critical during the pathogenesis of hyperoxia-induced lung injury. The Journal of Immunology, 2004, 172: 3860–3868.

Management of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) consists of low tidal volume protective ventilation, treatment of the inciting cause, prevention of nosocomial infections, and the administration of high concentrations of inspired oxygen with variable amounts of positive end-expiratory pressure (1, 2). The high concentrations of inspired oxygen used during the management of ALI/ARDS may result in increased reactive oxygen metabolites in the lung that may perpetuate the inflammatory response (3, 4).

Chemokines are 8- to 10-kDa proteins with 20–70% homology in amino acid sequences that are subdivided into families based on the relative position of the cysteine residues in the mature protein. Murine CXCL1 and CXCL2/3 are glutamic acid-leucine-arginine-positive (ELR-positive) CXC chemokines; are structural homologues of human growth-related oncogene-α (CXCL1) and growth-related oncogene-β/γ (CXCL2/3); and act as functional homologues to other human ELR-positive CXC chemokines in the mouse (5–9). Both murine chemokines share the ability to signal through a G protein-coupled receptor, CXCR2, and are potent neutrophil chemotactants and promote angiogenesis (5–7). Their human structural and functional homologues have been associated with ALI/ARDS (10–17). We hypothesized that the neutrophil recruitment to the lung during hyperoxia-induced lung injury is due, in part, to the expression of CXCR2 ligands through their interaction with their shared receptor, CXCR2.

Our study demonstrates that high concentrations of inspired oxygen lead to lung neutrophil sequestration and injury that parallel the expression of CXCL1 and CXCL2/3, and the recruitment of cells expressing CXCR2. Moreover, inhibition of CXCR2/CXCR2 ligand interaction markedly attenuates hyperoxia-induced lung injury and prolongs survival under hyperoxic conditions.

Materials and Methods

Murine model of hyperoxia-induced lung injury

Male C57BL/6 mice (6–8 wk) were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in specific pathogen-free conditions and provided with food and water ad libitum. To induce hyperoxia-induced lung injury, mice were allowed to roam free under normobaric pressures in 20-gallon chambers under varying concentrations of hyperoxia or room air to determine whether different concentrations of oxygen lead to differences in acute lung injury and survival. Oxygen mixtures or room air were delivered through the chamber at 3 liters/min and allowed to vent through a distal ventilation port to maintain normobaric pressures. In subsequent studies, mice were placed in 80% oxygen, and after 6 days of exposure, anesthetized with ketamine i.p.; then, under sterile conditions, the thorax was exposed. A 22-gauge needle was used to puncture the left atrium. A 22-gauge needle was introduced into the right ventricle, and 3 ml of PBS was infused at 20 cm H2O. The heart and thymus were removed and the lungs were dissected from the hilum. The tissue was frozen in liquid nitrogen and stored at −80°C for future processing and analysis. We also performed the same studies using CXCR2−/− mice on a C57BL/6 background, as compared with CXCR2+/− (wild-type) C57BL/6 mice.

Reagents

Biotinylated and nonbiotinylated anti-murine CXCL1 was purchased from R&D Systems (Minneapolis, MN). Polyclonal rabbit anti-murine CXCL2/3 used for ELISA was produced by the immunization of a rabbit with carrier-free murine rCXCL2/3 (R&D Systems) in multiple intradermal sites with CFA; followed by at least three boosts of CXCL2/3 in IFA, as previously described (18, 19).

Myeloperoxidase (MPO) assay

Pulmonary neutrophil sequestration was quantitated using a MPO assay, as previously described (20). Briefly, at the time of sacrifice, lungs were perfused free of blood with 3 ml of 0.9% saline via the spontaneously beating right ventricle. The lungs were excised from 10 mice under conditions of hyperoxia and from 6 mice under conditions of room air exposure and
placed in a 50 mM potassium phosphate buffer solution (pH 6.0) with 5% hexadecyltrimethylammonium bromide (Sigma-Aldrich, St. Louis, MO). The lung tissue was homogenized, sonicated, and centrifuged at 12,000 × g for 15 min at 4 °C. The supernatant was then assayed for MPO activity using a spectrophotometric reaction with o-dianisidine hydrochloride (Sigma-Aldrich) at 460 nm, as previously described (20).

**Histopathological grading of hyperoxia-induced lung injury**

Three random 5-μm paraffin-embedded tissue sections from five different lungs taken from the 80% oxygen-exposed and normal room air-exposed mice were stained with H&E at day 6. The histopathology was reviewed in a blinded manner with respect to which group or mouse was being reviewed, using a modified histologic scoring system, as previously described (7). Four easily identifiable pathological processes were scored on a scale of 0–4: 1) alveolar congestion, 2) hemorrhage, 3) leukocyte infiltration or aggregation of neutrophils in air space or the vessel wall, and 4) thickness of the alveolar wall. A score of 0 represented normal lungs; 1, mild, <25% lung involvement; 2, moderate, 25–50% lung involvement; 3, severe, 50–75% lung involvement; and 4, very severe, >75% lung involvement. An overall score of hyperoxia-induced lung injury was obtained based on the summation of all the scores, and then a mean ± SEM was generated from the cohort of normal room air- or hyperoxia-exposed lungs (three sections from each lung, six lungs per group) at each time point to generate a cumulative histological hyperoxia-induced lung injury score.

**Evans blue microvascular permeability and wet/dry analysis of lung edema**

Microvascular permeability related to lung injury was measured using a modification of the Evans blue dye extravasation technique, as previously described (20, 21). Extravasation of Evans blue (Sigma-Aldrich) into the extravascular compartment was used as a quantitative measure of lung injury and changes in pulmonary microvascular permeability. Briefly, each animal received 20 mg/kg Evans blue (pH 7.34) by tail vein injection 3 h before sacrifice. At the time of sacrifice, a heparinized sample of blood was harvested, and plasma was removed by centrifugation. Ten lungs from each group were perfused free of blood with 1 ml of 0.9% normal saline via the spontaneously beating right ventricle and removed from the thoracic cavity. The trachea, mainstem bronchi, and surrounding mediastinal structures were removed. Evans blue was extracted from pulmonary tissues after homogenization in 1 ml of 0.9% normal saline. This volume was added to 2 vol of deionized formamide and incubated at 60 °C for 12 h. The supernatant was separated by centrifugation at 2000 × g for 30 min. Evans blue in the plasma and lung tissue was quantitated by dual-wavelength spectrophotometric analysis at 620 and 740 nm (22). This method corrects the specimen’s absorbance at 620 nm for the absorbance of contaminating hemoglobin pigments, using the following formula: corrected absorbance at 620 nm = actual absorbance at 620 nm – (1.426 [absorbance at 740] + 0.03). We calculated a permeability index by dividing the corrected pulmonary tissue Evans blue absorbance at 620 μg/mL lung tissue by the corrected plasma Evans blue absorbance at 620 nm; this index reflects the degree of extravasation of Evans blue into the extravascular pulmonary tissue compartment.

To quantitate lung edema during hyperoxia-induced lung injury, we obtained wet/dry weight ratios by ligating 10 lungs per group away from the hilum. The lungs were blotted dry and weighed. They were then desiccated at 40 °C for 60 min at room temperature. The collagen-dye complex was precipitated by centrifugation at 5000 × g for 10 min and mixed with 1.0 ml of Sircol reagent; the absorbance was measured at 450 nm in an automated microplate reader (Bio-Tek Instruments, Winooski, VT). Standards were half-log dilutions of either CXCL1 or CXCL2/3 from 100 ng/ml to 1 pg/ml (50 μM/well). This ELISA method consistently detected specific chemokine concentrations greater than 50 pg/mg in a linear fashion. CXCL1 and CXCL2/3 were specific in our sandwich ELISA without cross-reactivity to a panel of cytokines, including human and murine IL-1 receptor antagonist, IL-2, IL-6, IL-8, TNF-α, IFN-γ, and members of the CXC and CC chemokine families.

**ELISA for phosphorylated I-kBα**

To assess activated NF-κB in tissue, phosphorylated I-kBα from whole lung homogenates of six mice per group was measured using a modification of a commercially available ELISA kit, I-kBα ActiELISA (BioCarta, San Diego, CA), as previously described (7). Briefly, a flat-bottom 96-well microtiter plate was coated with capture Ab for 24 h at 4 °C and then washed nonspecific binding sites were blocked with 2% BSA in PBS and incubated for 60 min at 37 °C. Plates were washed twice with wash buffer, and samples or standard were added, followed by incubation for 1 h at 37 °C. Plates were washed three times; 50 μl/well biotinylated anti-murine CXCL1 and CXCL2/3 Abs were added; and plates were incubated for 45 min at 37 °C. Plates were washed three times, and streptavidin-peroxidase conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA) was added; and the plates were incubated for 30 min at 37 °C. Plates were washed three times, and 3,3′,5,5′-tetramethylbenzidine chromogen substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added. The plates were incubated at room temperature to the desired extinction, and the reaction was terminated with 3 M H2SO4 solution. Plates were read at 450 nm in an automated microplate reader (BioTek Instruments). The standard curve was generated using half dilutions of phosphorylated I-kBα from 1000 μM/gl to 0, provided by the manufacturer (BioCarta).

**Total RNA isolation and RT-PCR amplification**

Total cellular RNA from lung tissue of six mice per group was isolated, as previously described (20, 23). Total RNA was transcribed into cDNA using the TaqMan Reverse Transcription kit (Applied Biosystems, Foster City, CA) and the cDNA was amplified and quantified using the TaqMan 7700 Sequence Detection System and specific primers for murine CXCL1, murine CXCL2/3, murine CXCR2, pro-IL-1α, pro-IL-6, pro-IL-10, pro-IL-12, and pro-TNF-α. The primers used were 5′-TGA-GCT-CTG-TCA-CTG-3′ (sense) and 5′-AGA-AGC-CGT-TGA-CCA-3′ (antisense) for CXCL1 (259 bp), 5′-GCT-GGG-CCG-CCA-GAG-3′ (sense) and 5′-GCC-GAT-CAT-GTA-GTC-3′ (antisense) for murine CXCL2/3 (359 bp); 5′-TCA-CTG-TGA-3′ (sense) and 5′-ACC-TCT-AGC-3′ (antisense) for pro-IL-1α (280 bp); and 5′-GCC-GAT-CAT-GTA-GTC-3′ (sense) and 5′-ACC-TCT-AGC-3′ (antisense) for pro-IL-6 (261 bp). Predeveloped assay reagents (Applied Biosystems kit 4304134) were used for murine CXCR2 and the housekeeping gene, 18S. The primers used were 5′-TGA-GCT-CTG-TCA-CTG-3′ (sense) and 5′-AGA-AGC-CGT-TGA-CCA-3′ (antisense) for CXCL1 (259 bp), 5′-GCT-GGG-CCG-CCA-GAG-3′ (sense) and 5′-GCC-GAT-CAT-GTA-GTC-3′ (antisense) for murine CXCL2/3 (359 bp); 5′-TCA-CTG-TGA-3′ (sense) and 5′-ACC-TCT-AGC-3′ (antisense) for pro-IL-1α (280 bp); and 5′-GCC-GAT-CAT-GTA-GTC-3′ (sense) and 5′-ACC-TCT-AGC-3′ (antisense) for pro-IL-6 (261 bp). Predeveloped assay reagents (Applied Biosystems kit 4304134) were used for murine CXCR2 and the housekeeping gene, 18S. Quantitative analysis of gene expression was done using the comparative C value (ΔC value) methods, in which C is the threshold cycle number (the minimum number of cycles needed before the product can be detected) (7). The arithmetic formula for the ΔC value method is the difference in threshold cycle numbers of a target gene and an endogenous reference (i.e., housekeeping gene 18S). The amount of target normalized to an endogenous reference (i.e., CXCR2) and relative to a calibration normalized to an endogenous reference (i.e., CXCR2 in room air controls) is given by 2ΔC−ΔC(7). The following is an example for comparing CXCR2 expression from hyperoxia-treated animals and room air controls. Both CXCR2 from the hyperoxia-treated and room air controls are normalized to 18S;
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DeltaCt - DeltaCt (CXCR2 expression from hyperoxia-treated animals normalized to endogenous 18S) - DeltaCt (CXCR2 expression from room air controls normalized to endogenous 18S). The calculation of 2-DeltaCt then gives a relative value when comparing the target with the calibrator, which we designate in this context as fold increase of hyperoxia-treated animals to room air controls of the target mRNA relative quantification.

FACS analysis of lung neutrophils

Whole lung single cell suspension preparations were made from harvested lungs from six mice per group using a method, as previously described (7). Single cell suspensions (5 x 10^6 cells/ml) were stained with primary Abs: Tricolor-conjugated (BD Biosciences, Franklin Lakes, NJ) anti-murine CD45 (Caltag Laboratories, South San Francisco, CA), anti-murine Ly-6G (neutrophil surface marker; BD Biosciences), or isotype controls. The secondary Ab used for these experiments was Alexa 488 (Molecular Probes, Eugene, OR). Dual-color-stained cell suspensions were analyzed on a FACSscan flow cytometer (BD Immunocytometry Systems, San Jose, CA) using CellQuest 3.2.1f1 software (BD Immunocytometry Systems).

Statistical analysis

Data were analyzed using the StatView 4.5 statistical package (Abacus Concepts, Berkeley, CA). Two group comparisons were evaluated using the unpaired t test. Three group comparisons were evaluated by the ANOVA test with the post hoc analysis (i.e., Bonferroni/Dunn). Survival was evaluated between groups using the Kaplan-Meier survival analysis and log rank test. Data were expressed as mean ± SEM.

Results

Oxygen induces mortality in a concentration-dependent manner

Because human studies have suggested that hyperoxia is associated with increased lung injury, we performed translational studies using a murine model system of hyperoxia-induced lung injury to dissect the mechanisms related to this process. Mice exposed to 100 and 90% oxygen became rapidly ill and demonstrated 100% mortality within 4–5 days (Fig. 1). Mice exposed to 80% oxygen showed 50% mortality by day 6 and 100% mortality within 12 days (Fig. 1). Finally, mice exposed to 70% oxygen remained mortality free during the study period of 14 days (Fig. 1).

Hyperoxia is associated with increased lung injury

Quantitative analysis of lung histopathology demonstrated more edema, leukocyte infiltration, alveolar hemorrhage, and alveolar wall thickness in lungs harvested at day 6 from the mice exposed.
to hyperoxia (80%) than mice exposed to room air (Fig. 2, IA and II). We found that lungs from the high oxygen concentration-exposed group had a significantly higher vascular permeability index than those mice exposed to room air (Fig. 2IB). These findings were confirmed by demonstrating a greater wet/dry ratio of lungs of animals exposed to 80% oxygen (Fig. 2IC). Furthermore, because lung injury during ARDS ultimately leads to extracellular matrix deposition, we evaluated both groups for collagen deposition in the lung. The lungs of mice exposed to 80% oxygen had more collagen deposition than the lungs of mice exposed to room air at day 6 (Fig. 2ID). Finally, histopathological analysis of lungs from the hyperoxia-exposed mice on day 6 demonstrated markedly increased alveolar congestion, alveolar hemorrhage, leukocyte infiltration, and alveolar wall thickness as compared with room air-exposed mice (Fig. 2II).

Neutrophils are recruited into the lung during hyperoxia-induced lung injury

Next, we measured neutrophil sequestration in the lung during 80% oxygen in comparison with normal room air-exposed control mice. C57BL/6 mice placed on either 80% oxygen or room air were sacrificed at 6 days, and their lungs were harvested for MPO analysis as an indirect measurement of neutrophil infiltration into the lung. Lung homogenates from the 80% oxygen-exposed group had significantly greater levels of MPO than lung homogenates of room air-exposed mice (Fig. 3A). FACS analysis of Ly-6G for neutrophils was used to confirm this finding by demonstrating a greater number of neutrophils infiltrating the lungs of hyperoxia-exposed mice when compared with the lungs of control mice (Fig. 3B).

**FIGURE 3.** Hyperoxia-induced neutrophil sequestration in the lung. A, Lung MPO levels from mice exposed to room air or 80% oxygen for 6 days (n = 10 mice per group; *, p < 0.05). B, FACS analysis of neutrophils from the lungs of mice exposed to either room air or hyperoxia for 6 days (n = 6 mice per group; *, p < 0.05).

**CXCL1 and CXCL2/3 mRNA expression and protein levels in the lung are elevated during hyperoxia-induced lung injury**

We found significantly higher levels of mRNA expression of CXCL1 and CXCL2/3 in the lungs from the 80% oxygen-exposed animals, as compared with the lung homogenates of room air-exposed mice (Fig. 4A and B, respectively). The expression of these chemokines paralleled lung neutrophil infiltration and hyperoxia-induced lung injury (Figs. 2 and 3). This was further confirmed by measuring protein levels of CXCL1 and CXCL2/3 in whole lung homogenates from mice exposed to 80% oxygen and room air. There were significantly higher protein levels of CXCL1 and CXCL2/3 in the lung homogenates from mice exposed to 80% oxygen as compared with lungs from mice exposed to room air (Fig. 4C and D, respectively). To determine that the elevated expression of CXCL1 and CXCL2/3 that resulted in neutrophil infiltration and hyperoxia-induced lung injury was compartmentalized to the lung and not a result of systemic increases in plasma CXCL1 and CXCL2/3, we next measured protein levels of CXCL1 and CXCL2/3 in plasma from mice exposed to 80% oxygen and room air. There were similar protein levels of CXCL1 and CXCL2/3 in the plasma from mice exposed to 80% oxygen as compared with lungs from mice exposed to room air (Fig. 4E and F, respectively).

**Expression of CXCL1 and CXCL2/3 is associated with increased phosphorylated I-κBα in the lung under hyperoxic conditions**

To begin to determine a potential mechanism for the increased gene expression of CXCL1 and CXCL2/3 in the lung under hyperoxic conditions, we assessed the presence of phosphorylated I-κBα. The analysis of the phosphorylation of I-κBα correlates with nuclear localization and activation of NF-κB (24–26). Lung homogenates of mice were analyzed at 6 days of continuous exposure to 80% oxygen as compared with room air-exposed mice. We found significantly higher levels of phosphorylated I-κBα in the lungs from the 80% oxygen-exposed animals than in the room air-exposed mice (Fig. 5).

**CXCR2 expression is increased during hyperoxia-induced lung injury**

The finding of increased levels of CXCL1 and CXCL2/3 associated with neutrophil sequestration and hyperoxia-induced lung injury led us to evaluate the expression of CXCR2 mRNA in the lungs of these animals. Lung homogenates from the 80% oxygen-exposed group had significantly greater expression of CXCR2 mRNA than lung homogenates of room air-exposed mice (Fig. 6). The expression of CXCR2 mRNA paralleled its ligand expression, neutrophil sequestration, and hyperoxia-induced lung injury (Figs. 2–4).

**CXCR2<sup>−/−</sup> mice display reduced neutrophil sequestration in the lung under conditions of hyperoxia**

With CXCL1 and CXCL2/3 correlating with the recruitment of neutrophils and expression of CXCR2, we next used a genetic strategy using C57BL/6 CXCR2<sup>−/−</sup> vs C57BL/6 CXCR2<sup>+/+</sup> mice, and compared their responses under conditions of 80% oxygen exposure at day 6. We found that lungs from CXCR2<sup>−/−</sup> mice had lower levels of MPO under conditions of 80% oxygen exposure at day 6, as compared with CXCR2<sup>+/+</sup> mice exposed to the same concentration of oxygen (Fig. 7). Furthermore, the levels of MPO from CXCR2<sup>−/−</sup> mice were not significantly different from the CXCR2<sup>+/+</sup> mice exposed to room air (Fig. 7).
CXCR2−/− mice were protected from hyperoxia-induced lung injury

Quantitative analysis of lung histopathology confirmed that CXCR2−/− mice were protected from hyperoxia-induced lung injury and indistinguishable from CXCR2+/+ mice exposed to room air (Fig. 8A). Both markers of lung microvascular permeability and edema confirmed that the lungs from CXCR2−/− mice retained their microvascular integrity under conditions of 80% oxygen at day 6 when compared with CXCR2+/+ mice exposed to the same conditions (Fig. 8I, B and C). In addition, CXCR2−/− mice exposed to 80% oxygen for 6 days demonstrated reduced collagen deposition in the lungs, as compared with CXCR2+/+ mice exposed to similar conditions, and the levels were similar to CXCR2+/+ exposed to room air (Fig. 8D). Histopathological analysis of lungs from the CXCR2−/− mice, as compared with CXCR2+/+ mice on day 6, demonstrated minimal alveolar con-

FIGURE 4. Hyperoxia induces the expression of CXCL1 and CXCL2/3 mRNA and protein in the lung. A and B, Quantitative levels of CXCL1 and CXCL2/3 mRNA, respectively, in the lungs from mice exposed to either room air or hyperoxia for 6 days (n = 6 mice per group; *, p < 0.05). C and D, Quantitative levels of CXCL1 and CXCL2/3 protein in the lungs, respectively, from mice exposed to either room air or hyperoxia for 6 days (n = 6 mice per group; *, p < 0.05). E and F, Quantitative levels of CXCL1 and CXCL2/3 protein, respectively, in plasma from mice exposed to either room air or hyperoxia for 6 days (n = 6 mice per group; no statistical difference found).

FIGURE 5. Hyperoxia induces increased levels of phosphorylated IκBα. Lung injury is associated with increased levels of NF-κB. Phosphorylated IκBα was measured from whole lung homogenates in mice exposed to either room air or hyperoxia for 6 days (n = 6 mice per group; *, p < 0.05).

FIGURE 6. Hyperoxia induces increased expression of CXCR2 mRNA in the lung. Quantitative RT-PCR was determined by Taqman analysis for CXCR2 mRNA from the lungs of mice exposed to either room air or 80% hyperoxia for 6 days (n = 6 mice per group; *, p < 0.05).
gestion, alveolar hemorrhage, leukocyte infiltration, and alveolar wall thickness, as compared with the lungs of CXCR2−/− mice exposed to 80% oxygen (Fig. 8II).

To determine whether the protective effects of CXCR2 depletion on lung injury were relevant to clinical survival, we compared the survival of CXCR2−/− mice with CXCR2+/+ mice under conditions of 80% oxygen exposure. CXCR2−/− mice exposed to 80% oxygen became ill and reached 100% mortality within 8 days. In contrast, CXCR2−/− mice exposed to 80% oxygen remained healthy and mortality free out to day 21 (Fig. 9; p < 0.0001).

**Discussion**

Acute lung injury is characterized by an initial microvascular leak with a neutrophil-predominant inflammatory response that promotes diffuse alveolar damage (1, 27). High concentrations of oxygen may perpetuate lung injury and result in persistent inflammation indistinguishable from that seen in ARDS (28–30). Hyperoxia-induced lung injury is characterized by noncardiogenic pulmonary edema, alveolar hyaline membrane formation, type I alveolar epithelial cell injury, type II alveolar epithelial cell hyperplasia, neutrophil infiltration, alveolar hemorrhage, and increased alveolar wall thickness (31, 32). As the lung injury progresses, the interstitium becomes infiltrated with leukocytes,
followed by pulmonary vascular remodeling and eventual development of fibrosis (33). Hyperoxia-induced lung injury is mediated through the generation of reactive oxygen species that may initiate and control the activation of NF-κB and its subsequent gene products such as CXC chemokines (34). In this study, we hypothesized that the interaction between CXC chemokines and ELR-positive CXC chemokines expressed under conditions of hyperoxia is critical in mediating neutrophil recruitment, a pivotal process required for hyperoxia-induced lung injury.

Previous studies have demonstrated that mice exposed to 100% oxygen rapidly become moribund and reach uniform mortality within 3 days (35, 36). This present study extends these findings by determining the concentration-dependent effect of hyperoxia-induced lung injury on mortality. Because of our interest in the pathophysiological mechanisms leading to hyperoxia-induced lung injury, the time point and oxygen concentration in which the mice had significant lung injury, yet no overwhelming mortality, were focused upon. Histopathological analysis demonstrated increased lung injury, consistent with other animal studies of hyperoxia-induced lung injury (37, 38). In addition, we further characterized the alveolar-capillary membrane injury for pulmonary edema and measurements of collagen deposition. Interestingly, our findings support the work of previous investigators who demonstrated that increased levels of procollagen III in bronchoalveolar lavage fluid were a marker of acute lung injury and early mortality (39, 40).

Having characterized the histopathological damage caused by hyperoxia, we then focused on the underlying mechanisms responsible for promoting the inflammation and subsequent lung injury. Our findings of a significant increase in neutrophil infiltration in hyperoxia-exposed mice complement and extend the conclusions of other studies that found that high concentrations of oxygen led to the release of reactive oxygen species that promoted neutrophil infiltration into the lung (41, 42). Lungs taken from multiple species after 24 h of hyperoxia, when studied by electron microscopy, demonstrate a diffuse infiltration of activated neutrophils (43). In addition, increases in P-selectin and up-regulation in ICAM-1, a ligand for neutrophil β2 integrins, have been demonstrated early in the course of hyperoxia exposure, lending further evidence for a neutrophil-mediated injury (44–47). This exemplifies that hyperoxia by itself can lead to increased neutrophil infiltration. However, the molecular and cellular mechanisms involved in recruiting these neutrophils remain to be fully elucidated.

Elegant in vitro studies have demonstrated that either hyperoxia or oxidant stress can induce IL-8 expression from human alveolar macrophages, endothelial cells, and epithelial cells (48, 49). These findings suggest that high concentrations of oxygen can induce the expression of CXC chemokines by multiple cell types in the lung, resulting in increased neutrophil infiltration and hyperoxia-induced lung injury as characterized by increased vascular permeability, pulmonary edema, and fibrosis. In our study, we did not find any significant elevations in the plasma levels of CXC chemokines between groups, suggesting that CXCL1 and CXCL2/3 are up-regulated in a local compartmentalized manner in the lung.

Having demonstrated that hyperoxia is associated with increased neutrophil sequestration, we determined that CXCL1 and CXCL2/3 expression was significantly greater in the lungs of the 80% oxygen-exposed mice than in the lungs of room air controls. These results are similar to, and extend the findings of Deng et al. (50), who found elevated levels of CXCL1 and CXCL2/3 in 95% oxygen-exposed lungs in neonatal rats after 4 days of exposure. The levels of CXCL1 and CXCL2/3 in both our study and that of Deng et al. (50) demonstrate that these CXC chemokines are expressed under conditions of hyperoxia and are associated with the presence of neutrophils in the lung. In contrast to our study, Deng et al. (50) used a strategy of neutralizing either CXCL1 or CXCL2/3. Although this strategy resulted in a marked reduction of neutrophils in the bronchoalveolar lavage fluid, it did not suppress the levels of lung tissue MPO to the same magnitude that we see in the CXCR2−/− mice under similar conditions. The magnitude of reduction of MPO in our model system using CXCR2−/− mice under hyperoxic conditions may reflect that we attenuated the effect of not only CXCL1 and CXCL2/3, but all ELR-positive CXC chemokines that may have been expressed under these conditions and use the same shared receptor.

For example, in a study by Auten et al. (51), they only used neutralizing Abs to CXCL1 in the same rat newborn model of hyperoxia as Deng et al. (50). In this study, they found a marked reduction of neutrophils in the bronchoalveolar lavage fluid in response to hyperoxia and neutralization of CXCL1 (51). However, the use of the neutralizing anti-CXCL1 Abs did not fully attenuate levels of CXCL1 in the bronchoalveolar lavage fluid, nor did this approach fully protect the lung in hyperoxia-exposed animals (51), suggesting that the effect seen was related to the inability to fully deplete CXCL1 in their system. In our study, the more profound protective effect on parameters of lung injury under conditions of hyperoxia in the CXCR2−/− mice, as compared with CXCR2+/− mice, is due to the total loss of CXCR2/CXCR2 ligand biology.

NF-κB is a transcription factor that can modulate the expression of cytokines and chemokines during cellular stress, and has been implicated in multiple inflammatory processes in the lung (52, 53). Phosphorylation of IκBα and degradation of IκBα protein have been shown to correlate with NF-κB activation and transcription of CXC chemokines (24, 25). Having found that hyperoxia-induced lung injury is mediated through CXCL1- and CXCL2/3-induced neutrophil infiltration, we demonstrated an increase in phosphorylation of IκBα in the mice exposed to 80% oxygen at day 6, as compared with mice exposed to room air. Others have found that NF-κB is up-regulated in response to hyperoxia in both in vitro and ex vivo lung preparations (54–57). Our study extends these findings by suggesting that hyperoxia promotes phosphorylation of IκBα that may be associated with activation of NF-κB, resulting in the subsequent expression of CXCL1 and CXCL2/3, which leads to increased neutrophil infiltration and subsequent lung injury.

The expression of CXCR2 mRNA paralleled the production of both CXCL1 and CXCL2/3 ligands and neutrophil sequestration during hyperoxia-induced lung injury. Other studies of inflammatory diseases, such as ventilator-induced lung injury and pneumonia, have demonstrated the importance of CXCR2 expression and its role in neutrophil recruitment during the pathogenesis of these diseases (7, 18, 58). Collectively, these studies demonstrate that
augmented levels of CXCR2 ligands are important in the recruitment of neutrophils during the pathogenesis of inflammatory diseases and suggest that the interaction between CXCR2 ligands and CXCR2 may be pivotal in the recruitment of neutrophils to the lung during hyperoxia-induced lung injury.

Based on the above findings, we performed proof of principle studies in vivo using a genetic approach to evaluate the direct role for CXCL1 and CXCL2/3 ligands and their interaction with studies in vivo using a genetic approach to evaluate the direct role of the lung during hyperoxia-induced lung injury. The CXCR2+/− mice placed in 80% oxygen demonstrated significant reductions in neutrophil infiltration that were paralleled by a decrease in lung injury. Our findings corroborate and extend the findings of Auten et al. (58), who reported that neutrophil accumulation was prevented through the use of a nonpeptide CXCR2 antagonist in newborn rats under conditions of 95% oxygen. However, this study did not demonstrate the impact of this treatment on parameters of lung injury.

Although previous studies have shown that using nonpeptide CXCR2 antagonist or neutralizing CXCL1 or CXCL2/3 Abs can reduce neutrophil infiltration into the lung under hyperoxic conditions (50, 51, 58), our study is the first to demonstrate that knock-out of a single gene, CXCR2, can result in a marked survival advantage of adult mice in hyperoxia. This finding was supported by Kaplan-Meier survival analysis and log rank test, and was in contrast to studies using only a strategy of neutralization of CXCL1 for a limited period of time (50, 51). Moreover, our findings demonstrate that the interactions between CXCR2 ligands and CXCR2 are paramount to the generation of an inflammatory response characterized by neutrophil sequestration and alveolar-capillary membrane injury in the adult lung. Taken together, these findings highlight the importance of CXCR2 ligand/CXCR2 interaction in neutrophil recruitment and activation during the pathogenesis of hyperoxia-induced lung injury.

In conclusion, we have demonstrated that the biological axis of CXCR2 ligand/CXCR2 signaling plays a pivotal role in mediating hyperoxia-induced lung injury. In contrast to studies using only a strategy of neutralization of CXCL1 in hyperoxia. This advantage of adult mice in hyperoxia. This findings of the current study support the contention that CXCR2 ligands and CXCR2 mediate hyperoxia-induced lung injury and mortality, and may be a therapeutic target to attenuate this pathology.

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