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CXC Chemokine Receptor-2 Ligands Are Necessary Components of Neutrophil-Mediated Host Defense in Invasive Pulmonary Aspergillosis

Borna Mehrad*, Robert M. Strieter, Thomas A. Moore, Wan C. Tsai, Sergio A. Lira†, and Theodore J. Standiford2*

Invasive pulmonary aspergillosis is a devastating complication of immunosuppression, which occurs in association with neutrophil dysfunction or deficiency. ELR+ CXC chemokines are a subfamily of chemokines that play a critical role in neutrophil chemotaxis and activation both in vitro and in vivo. We hypothesized that interaction of these ligands with CXC chemokine receptor-2 (CXCR2), their sole murine receptor, is a major component of neutrophil-dependent pulmonary host defense against Aspergillus fumigatus. In immunocompetent animals, neutrophils were recruited to the lung in response to intratracheally administered A. fumigatus conidia. In a model of transient in vivo depletion of neutrophils, animals developed invasive pulmonary aspergillosis, associated with delayed influx of neutrophils into the lung. In both normal and neutrophil-depleted animals, the ELR+ CXC chemokines MIP-2 and KC were induced in response to intratracheal administration of conidia. Ab-mediated neutralization of the common ELR+ CXC chemokine receptor, CXCR2, resulted in development of invasive disease indistinguishable from the disease in neutrophil-depleted animals, while control animals were highly resistant to the development of infection. CXCR2 neutralization was associated with reduced lung neutrophil influx and resulted in a marked increase in mortality compared with controls. In contrast, animals with constitutive lung-specific transgenic expression of KC were resistant to the organism, with reduced mortality and lower lung burden of fungus. We conclude that CXCR2 ligands are essential mediators of host defense against A. fumigatus conidia, and may be important targets in devising future therapeutic strategies in this disease.  


Invasive pulmonary aspergillosis is a common and devastating complication of immunosuppression. Evidence suggests an alarming recent increase in the incidence of this disease, which has been attributed to the increasing prevalence of immunocompromised patients (1–3). Current therapy for this infection is limited by a poor rate of response (4, 5), such that the mortality of invasive pulmonary aspergillosis is >85% despite best available treatment (6).

Invasive aspergillosis has been noted to occur in the setting of agranulocytosis since its original description (7), and deficiencies in neutrophil number or function have since been recognized to be strongly associated with the development of invasive aspergillosis in diverse populations of patients (8–11). In vitro studies have shown that normal neutrophils efficiently damage Aspergillus hyphae (12, 13). In contrast, dysfunctional neutrophils, such as those treated with corticosteroids or those isolated from patients with myeloperoxidase (MPO)3 deficiency or chronic granulomatous disease are inefficient at killing Aspergillus hyphae (14, 15). In vivo animal studies have established a causative relationship between neutrophil dysfunction or deficiency and the development of invasive aspergillosis (16, 17). However, factors that regulate neutrophil recruitment and activation in invasive aspergillosis have not been characterized.

CXC chemokines are a family of related 8- to 10-kDa polypeptide molecules that are potent chemotactic factors for numerous populations of immune cells and are characterized by four conserved cysteine residues at the N terminus, the first two of which are separated by a nonconserved amino acid. The CXC chemokines are further subdivided into those that contain the amino acid sequence glutamic acid-leucine-arginine (the ELR motif) immediately preceding the CXC sequence, and those that do not. The human ELR+ CXC chemokines include IL-8, the growth-related oncogene (GRO) chemokines, epithelial neutrophil-activating protein-78 (ENA-78), neutrophil-activating peptide-2 (NAP-2), and granulocyte chemotactic peptide-2 (GCP-2). Murine ELR+ CXC chemokines include macrophage inflammatory protein-2 (MIP-2), KC, GCP-2, LPS-induced CXC chemokine (LIX), and Lungkine (18, 19). ELR+ CXC chemokines have been shown to mediate neutrophil chemotaxis and activation in vitro (20–24) and in diverse in vivo settings (25–27). Several ELR+ CXC chemokines have been shown to be expressed in murine models of bacterial pneumonia (28), but the neutralization of specific chemokines has not altered survival dramatically (29).

Two human ELR+ CXC chemokine receptors have been identified, and are designated CXC chemokine receptor-1 (CXCR1) and CXC chemokine receptor-2 (CXCR2). These receptors are exclusively expressed on the surface of human granulocytes, and are

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3 Abbreviations used in this paper: MPO, myeloperoxidase; CCR1, CC chemokine receptor-1; CXCR2, CXC chemokine receptor-2; GMS, Gomori methanamine silver; ELR, glutamic acid-leucine-arginine; ENA-78, epithelial neutrophil activating protein-78; GCP-2, granulocyte chemotactic protein-2; GRO, growth-related oncogene; H&E, hematoxylin and eosin; I.t., intratracheal; LIX, LPS-induced CXC chemokine; MIP-1α, macrophage inflammatory protein-1α; MIP-2, macrophage inflammatory protein-2; NAP-2, neutrophil activating peptide-2; BAL, bronchoalveolar lavage; GMS, Gomori methanamine silver.
involved in neutrophil chemotactic and degranulation responses (30–32). CXCR1 binds IL-8 and GCP-2, while CXCR2 binds all ELR+ CXC chemokines with equal avidity (33, 34). The CXCR1 is not expressed in the mouse, but the murine CXCR2 is highly homologous to human CXCR2. Like its human counterpart, murine CXCR2 is promiscuous and has been shown to bind to several murine ELR+ CXC chemokines. Neutrophils from mice with targeted deletion of CXCR2 fail to respond to macrophage inflammatory protein-2 (MIP-2) or KC in vitro, but exhibit normal chemotaxis to C5a (35). In vivo studies, CXCR2 knockout mice fail to develop tissue neutrophilic infiltrates in response to s.c. urate crystals (36), suggesting that ELR+ CXC chemokines are the major mediator of neutrophil influx in this model, and that CXCR2 is the sole receptor for these ligands. The role of CXCR2 in the setting of infections has not been examined. The presence of multiple ligands and the promiscuity of the receptors have made the setting of infections has not been examined. The presence of multiple ligands and the promiscuity of the receptors have made the study of human ELR+ CXC chemokines difficult. In this context, the mouse system, which lacks CXCR1, provides a simplified system for studying the ligand-receptor interaction of ELR+ CXC chemokines.

In this study, we hypothesized that the interaction of ligands with CXCR2 is a major component of neutrophil-dependent pulmonary host defense against A. fumigatus by mediating neutrophil deployment in response to the organism. To test this hypothesis, we evaluated the outcome and severity of infection in the setting of CXCR2 neutralization, and in the setting of compartmentalized overexpression of KC in the lung.

### Materials and Methods

#### Reagents

Polyclonal antiserum MIP-2 and KC Abs used in the ELISAs were produced by immunization of rabbits with carrier-free murine MIP-2 and KC (R&D Systems, Minneapolis, MN) in multiple intradermal sites with CFA, as previously described (37, 38). Polyvalent goat antiserum CXCR2 Abs used in vivo neutralization studies were produced by immunization of a goat with murine rCXCR2 peptide in multiple intradermal sites with CFA. The peptide sequence, Met-Gly-Glu-Phe-Lys-Val-Asp-Lys-Phe-Val, is a portion of the seven transmembrane receptor that resides on the cell surface and has previously been shown to be the binding site for ligands (39). Abs were purified over a protein A column and endotoxin contamination excluded by Limulus lystate assay (ICN Biomedical, Costa Mesa, CA). In CXCR2 neutralization experiments, 0.5 ml goat antiserum CXCR2 serum, or control goat serum, was administered i.p. 2 h prior to A. fumigatus administration. In preliminary studies, administration of this Ab did not affect the number of circulating neutrophils over the subsequent 4 days (data not shown). In separate experiments, administration of anti-CXCR2 abrogated the influx of neutrophils into the peritoneum of normal mice in response to exogenous KC (data not shown).

#### Animals

Specific pathogen-free C57Bl/6 mice (6 to 8-wk-old females, The Jackson Laboratories, Bar Harbor, ME) were used in all experiments except those involving KC transgenic mice. Specific pathogen-free transgenic KC mice C57Bl/6 (6 to 8-wk-old females) were generated on a B6D2 background by microinjection of fertilized eggs carrying the KC transgene into the C57Bl/6 genome. The organism was grown on Sabouraud dextrose agar plates (Becton Dickenson, Cockeysville, MD) for 7 to 10 days at 37°C. The surface of each plate was then washed with 100 ml of sterile 0.1% Tween 80 (SigmaUltra, St. Louis, MO) in normal saline. The resulting suspension of conidia was filtered through sterile gauze to remove clumps and hyphal debris, and then washed once and resuspended in 4 ml of 0.1% Tween-80. The concentration of Aspergillus conidia in the suspension was determined by a particle counter (Z2 particle analyzer, Coulter, Hialeah, FL). The suspension was then diluted to the desired concentration, and the concentration was again measured prior to administration.

In preliminary experiments, the number of particles determined by the particle counter was in close agreement with the number of viable CFUs found by serial dilution and plating of the suspension. On the day of inoculation, each animal was anesthetized with 1.8–2 mg pentobarbital i.p. Using standard aseptic technique, the trachea was exposed and a 30-μl inoculum (A. fumigatus suspension or 0.1% Tween-80) was administered via a sterile 26-gauge needle. The skin incision was closed with surgical staples. Animals were challenged with inocula ranging from 1–2 × 10³ to 1–2 × 10⁵ conidia in various experiments.

#### Lung harvest

At designated time points, the mice were sacrificed by CO₂ asphyxiation. The chest cavity was opened aseptically, and the pulmonary vasculature was perfused with PBS via the right ventricle. For histologic examination, lungs were perfused with 1 ml 4% paraformaldehyde in PBS, inflated with 1 ml 4% paraformaldehyde in PBS via the trachea, and then excised en bloc. Lungs for various assays were perfused with 1 ml of PBS containing 5 mM EDTA, removed, frozen in liquid nitrogen, and stored at −80°C until the day of the assay. Lungs for cytokine and MPO assays were homogenized in 1 ml of 2% complete protease inhibitor cocktail buffer (Boehringer Mannheim, Mannheim, Germany) in PBS, using a tissue homogenizer (Biospec Products, Bartlesville, OK). A 900-μl aliquot of PBS was added to 900 μl from each sample, sonicated for 10 s, and centrifuged at 5000 × g for 10 min. Supernatants were passed through a 0.45-micron filter (Gelman Sciences, Ann Arbor, MI), and stored at 4°C for cytokine ELISA.

#### Lung chitin assay

Given that molds (including Aspergillus species) do not reliably form reproductive units in tissue, we employed an assay for chitin to measure the burden of organisms in lungs. Chitin is a component of the hyphal wall that is absent from mammalian cells and conidia. The assay was adapted from a previously described method, which demonstrated a direct correlation between the weight of hyphae and level of chitin, as detected by assay (49). Lungs were homogenized in 5 ml distilled water and centrifuged (1500 × g, 5 min, 20°C). The supernatants were discarded, pellets resuspended in sodium laurel sulfate (3% w/v), and heated at 100°C for 15 min. Samples were then centrifuged (1500 × g, 5 min, 20°C), pellets washed with distilled water, and resuspended in 3 ml KOH (120°C, 15 min), heated at 130°C for 60 min. After cooling, 8 ml of ice-cold ethanol (75% v/v) was added to each sample, and tubes were shaken until ethanol and KOH made one phase. Samples were incubated on ice for 15 min, and 0.3 ml of Celite suspension (supernatant of 1 g of Celite 545 (Fisher Scientific, Pittsburgh, PA) added to 75% ethanol and allowed to stand for 2 min) was added to each. Samples were centrifuged (1500 × g, 5 min, 4°C), and supernatants were discarded. Pellets were washed once with ethanol (40% v/v) and twice with distilled water, and resuspended in 0.5 ml distilled water. Standards, consisting of 0.2 ml distilled water and 0.2 ml glucosamine (10 μg/ml), were made up. A total of 0.2 ml NaNO₃ (5% w/v) and 0.2 ml KHSO₃ (5% w/v) was added to each standard, and 0.5 ml NaNO₃ (5% w/v) and 0.5 ml KHSO₃ (5% w/v) was added to each tissue preparation; all samples were mixed gently for 15 min, and then centrifuged (1500 × g, 2 min, 4°C). Two 0.6-ml aliquots of supernatant from each tissue preparation were transferred to separate tubes. A total of 0.2 ml ammonium sulfamate was added to each tube, and all tubes were shaken

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Preparation and administration of A. fumigatus conidia

We chose to use A. fumigatus strain 13073 (American Type Culture Collection, Manassas, VA) in our studies, as this strain has previously been shown to induce invasive aspergillosis in immunocompromised mice (48). The organism was grown on Sabouraud dextrose agar plates (Becton Dickenson, Cockeysville, MD) for 7 to 10 days at 37°C. The surface of each plate was then washed with 100 ml of sterile 0.1% Tween 80 (SigmaUltra, St. Louis, MO) in normal saline. The resulting suspension of conidia was filtered through sterile gauze to remove clumps and hyphal debris, and then washed once and resuspended in 4 ml of 0.1% Tween-80. The concentration of Aspergillus conidia in the suspension was determined by a particle counter (Z2 particle analyzer, Coulter, Hialeah, FL). The suspension was then diluted to the desired concentration, and the concentration was again measured prior to administration. In preliminary experiments, the number of particles determined by the particle counter was in close agreement with the number of viable CFUs found by serial dilution and plating of the suspension. On the day of inoculation, each animal was anesthetized with 1.8–2 mg pentobarbital i.p. Using standard aseptic technique, the trachea was exposed and a 30-μl inoculum (A. fumigatus suspension or 0.1% Tween-80) was administered via a sterile 26-gauge needle. The skin incision was closed with surgical staples. Animals were challenged with inocula ranging from 1–2 × 10³ to 1–2 × 10⁵ conidia in various experiments.
vigorously for 5 min. A fresh solution of 3-methyl-2-thiazolone hydratzone HCl monohydrate (50 mg in 10 ml distilled water) was made, and 0.2 ml was added to each tube. Samples were then heated to 100°C for 3 min and cooled. A total of 0.2 ml of FeCl₃•6H₂O (0.83% w/v) was added to each, and OD was measured at 650 nm after 25 min. Chitin content, measured in glucosamine equivalents, was measured by the following formula: chitin content = {[(5 × (OD of organ – OD of control organ))/OD of glucosamine – OD of water]}.  

Lung MPO activity

Lung MPO activity was measured as a marker of neutrophil sequestration, as described previously (50). Briefly, a 100-μl aliquot of each lung homogenate was added to 100 μl of a buffer containing 50 mM potassium phosphate (pH 6.0), 5% hexadecyltrimethylammonium bromide, and 5 mM EDTA. Samples were sonicated for 10 s and centrifuged at 3000 g for 16 h at 4°C and then washed with PBS, pH 7.5, 0.05% Tween-20 (wash buffer). Microtiter plate nonspecific-binding sites were blocked with 2% BSA in PBS and incubated for 90 min at 37°C. Plates were rinsed four times with wash buffer, and diluted (neat and 1:10) cellfree supernatants (50 μl) in duplicate were added, followed by incubation for 1 h at 37°C. Plates were washed four times, followed by the addition of 50 μl/well biotinylated rabbit anti- cytokine Abs (3.5 μg/ml in PBS, pH 7.5, 0.05% Tween-20, and 2% FCS), and plates incubated at 37°C. Plates were washed four times. Streptavidin-peroxidase conjugate (Bio-Rad, Richmond, CA) was added, and the plates were incubated for 30 min at 37°C. Plates were washed again four times and chromogen substrate (Bio-Rad) was added. The plates were incubated at room temperature to the desired extinction, and the reaction was terminated with 50 μl/well of 3M H₂SO₄ solution. Plates were read at 940 nm in an ELISA reader. Standards were 1/2 log dilutions of recombinant murine MIP-2 or KC, from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected the relevant cytokine at concentrations above 25 pg/ml in PBS, pH 7.5, 0.05% Tween-20, and 2% FCS. MPO units were calculated as the change in absorbency over time.

Chemokine ELISA

Murine MIP-2 and KC levels were quantified using a modification of a double-ligand method, as described previously (51). Briefly, flat-bottom 96-well microtiter plates (Immuno-Plate I 96-F, Nunc Roskilde, Denmark) were coated with 50 μl/well of rabbit anti-cytokine Ab (1 μg/ml in 0.6 M NaCl, 0.26 M H₃BO₄, and 0.08 N NaOH, pH 9.6) for 16 h at 4°C and then washed with PBS, pH 7.5, 0.05% Tween-20 (wash buffer). Microtiter plate nonspecific-binding sites were blocked with 2% BSA in PBS and incubated for 90 min at 37°C. Plates were rinsed four times with wash buffer, and diluted (neat and 1:10) cellfree supernatants (50 μl) in duplicate were added, followed by incubation for 1 h at 37°C. Plates were washed four times, followed by the addition of 50 μl/well biotinylated rabbit anti- cytokine Abs (3.5 μg/ml in PBS, pH 7.5, 0.05% Tween-20, and 2% FCS), and plates incubated at 30 min at 37°C. Plates were washed four times. Streptavidin-peroxidase conjugate (Bio-Rad, Richmond, CA) was added, and the plates were incubated for 30 min at 37°C. Plates were washed again four times and chromogen substrate (Bio-Rad) was added. The plates were incubated at room temperature to the desired extinction, and the reaction was terminated with 50 μl/well of 3M H₂SO₄ solution. Plates were read at 940 nm in an ELISA reader. Standards were 1/2 log dilutions of recombinant murine MIP-2 or KC, from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected the relevant cytokine at concentrations above 25 pg/ml. The ELISAs did not cross-react with IL-1, IL-2, IL-4, IL-6, IL-10 or nont murine MIP-2 or KC, from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected the relevant cytokine at concentrations above 25 pg/ml. The ELISAs did not cross-react with IL-1, IL-2, IL-4, IL-6, IL-10 or nont murine MIP-2 or KC, from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected the relevant cytokine at concentrations above 25 pg/ml. The ELISAs did not cross-react with IL-1, IL-2, IL-4, IL-6, IL-10 or nont murine MIP-2 or KC, from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected the relevant cytokine at concentrations above 25 pg/ml. The ELISAs did not cross-react with IL-1, IL-2, IL-4, IL-6, IL-10 or nont murine MIP-2 or KC, from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected the relevant cytokine at concentrations above 25 pg/ml.

Statistical analysis

Data were analyzed by a Power Macintosh 8600/300 computer using InStat version 2.01 statistical package (GraphPad Software, San Diego, CA). Survival data were compared using the Fisher’s exact test. All other data were expressed as mean ± SEM and compared using an unpaired two-tail Mann-Whitney (nonparametric) test. Probability values were considered statistically significant if they were less than 0.05.

Results

Influx of neutrophils into the lungs in response to A. fumigatus

We first determined the time course of neutrophil influx into the lungs of normal C57BL/6 mice in response to challenge with 1–2 × 10⁷ A. fumigatus conidia. Lungs were harvested at various time points after challenge for histology and measurement of MPO activity, a surrogate measure of neutrophil sequestration. Lung MPO levels were elevated over baseline at 8 h after inoculation, and reached a plateau at 24 h (Fig. 1). Histology showed evidence of a patchy peribronchial infiltration of inflammatory cells within both the interstitial and alveolar compartments at 24 h, which predominantly consisted of neutrophils, and to a lesser extent, of mononuclear cells (data not shown). The cellular infiltrate was resolving by day 4 after inoculation, and predominantly consisted of mononuclear cells (data not shown).

Effect of neutrophil depletion on host response to A. fumigatus

In order to assess the contribution of neutrophils to host defense against A. fumigatus, we next challenged neutrophil-depleted mice with i.t. conidia. Neutrophil depletion was achieved by i.p. administration of 100 μg of RB6-8C5 mAb i.p. 1 day before i.t. A. fumigatus. In preliminary studies, we found that administration of this dose of RB6-8C5 resulted in peripheral blood neutropenia (absolute circulating neutrophil count <50 cells/μl) by days 1 and 3 after Ab administration in both infected and control animals, with a restoration of peripheral counts to pretreatment levels by day 5; administration of control serum did not affect the peripheral counts or lung histology (data not shown). We found a dose-dependent mortality in mice pretreated with RB6-8C5, depending on the size of A. fumigatus inoculum (Fig. 2). Importantly, pretreatment with RB6-8C5 leads to greater than 90% mortality in mice challenged...
with $1.89 \times 10^6$ conidia, while no deaths occurred in control animals challenged with the same inoculum.

In order to characterize the host response to *A. fumigatus* in neutrophil-depleted animals, lungs were examined histologically at various time points after i.t. challenge with $1-2 \times 10^6$ conidia. We found evidence of patchy peribronchial infiltration of inflammatory cells within both the interstitial and alveolar compartments in both normal and RB6-8C5-treated animals (Figs. 3, A and C). The cellular infiltrate consisted predominantly of neutrophils in normal animals, and predominantly mononuclear cells in RB6-8C5-treated mice. Conidia were visible in normal mice on day 2 after inoculation, which were generally cleared by day 4 (Fig. 3B), whereas neutrophil-depleted animals, hyphae were noted on days 2 and 4 (Fig. 3D). In order to quantify the presence of the invasive hyphal form of *A. fumigatus*, we then measured the lung burden of chitin in neutrophil-depleted animals. Lung chitin levels were undetectable in control animals, but were elevated on both days 2 and 4 after inoculation of conidia in both normal animals and animals treated with RB6-8C5 as compared with animals challenged with vehicle. Both MIP-2 and KC reached higher levels in animals treated with RB6-8C5 as compared with normal animals (Fig. 6). MIP-2 and KC were undetectable in plasma in both normal and RB6-8C5-treated animals, indicating a compartmentalized response.

We next assessed the time course of neutrophil influx into the lungs of RB6-8C5-treated animals by measuring lung MPO content at various time points after inoculate of $1-2 \times 10^6$ conidia (Fig. 5A). Normal animals had maximal lung MPO levels 1 day after challenge, with gradual return to pretreatment levels over days 2 to 4. As compared with normal animals, RB6-8C5-treated animals had lesser quantities of lung MPO at day 1 after challenge. However, RB6-8C5-treated animals had increased levels of lung MPO by day 2 after challenge with *A. fumigatus*, as compared with immunocompetent animals. In order to correlate these findings with the number of cells present, we performed cell counts and differentials on fluid obtained by bronchoalveolar lavage 2 days after challenge with $1-2 \times 10^6$ conidia. Normal animals challenged with conidia had an increased number of recovered cells, which were predominantly neutrophils. RB6-8C5-treated animals had a greater total number of recovered cells, the majority of which were neutrophils (Fig. 5B). The numbers of neutrophils counted on bronchoalveolar lavage (BAL) in various experimental groups were in agreement with lung MPO levels measured on day 2.

Expression of ELR$^+$ CXC chemokines in response to *A. fumigatus*

To address the mechanism of neutrophil influx into the lungs in response to challenge with *A. fumigatus*, we next measured concentrations of the ELR$^+$ CXC chemokines MIP-2 and KC in lung homogenates at various times after i.t. administration of conidia. KC and MIP-2 levels were elevated 1 day after inoculation with conidia in both normal animals and animals treated with RB6-8C5 as compared with animals challenged with vehicle. Both MIP-2 and KC reached higher levels in animals treated with RB6-8C5 as compared with normal animals (Fig. 6). MIP-2 and KC were undetectable in plasma in both normal and RB6-8C5-treated animals, indicating a compartmentalized response.
To assess the contribution of the ELR<sup>1</sup>CXC chemokines to host response against <i>A. fumigatus</i>, we next assessed survival of animals pretreated with anti-CXCR2 Abs. In preliminary studies, administration of this Ab to normal animals did not affect the number of circulating neutrophils over the subsequent 4 days (data not shown). Administration of anti-CXCR2 serum to normal animals 2 h before challenge with 1–2×10<sup>7</sup> conidia resulted in a marked increase in mortality, with >90% mortality in animals treated with anti-CXCR2, as compared with minimal mortality in immunocompetent animals treated with a similar inoculum of <i>A. fumigatus</i> (Fig. 7).

In order to determine the cause of increased lethality in anti-CXCR2-treated animals, mice were sacrificed on day 2 after challenge with conidia, and lungs were harvested for histology, chitin content, and MPO activity. Lungs of animals treated with control serum contained a dense peribronchial, predominantly neutrophilic cellular infiltration, corresponding to areas of conidia deposition (Fig. 8, A and B). Hyphae were not present. However, lungs of animals treated with anti-CXCR2 serum contained patchy peribronchial mononuclear cellular infiltration and fibrin deposition, with a paucity of neutrophils as compared with anti-CXCR2-treated animals (Fig. 8, C). Importantly, fungal stains showed few conidia and large numbers of hyphae, indicating the development of invasive disease (Fig. 8, D).

To quantify the amount of hyphae present, lung chitin levels were measured 2 days after challenge with conidia, in animals receiving i.t. vehicle or anti-CXCR2 serum. A 30-fold increase in lung chitin content was noted in animals treated with anti-CXCR2 wild compared with control animals (Fig. 9A). Furthermore, lungs of animals treated with anti-CXCR2 contained 63% less myeloperoxidase at 2 days after challenge with <i>A. fumigatus</i>, as compared with animals treated with control serum (Fig. 9B), corroborating the histologic observation of paucity of neutrophils.

Effect of compartmentalized overexpression of KC on host defense against <i>A. fumigatus</i>

Having demonstrated the neutralization of the common receptor for ELR<sup>+</sup>CXC chemokines results in marked impairment of host
CXCR2, anti-CXCR2 serum. Experimentalpared with animals receiving control serum. Af, A. fumigatus experiments. representative of two experiments. Pooled data from two separate Animals were challenged with 1–2 peripheral counts to pretreatment levels by day 5 (data not shown). days 1 and 3 after injection in all animals, with restoration of RB6-8C5 mAb. animals was normal and was not affected by administration of after 24 h. Lung histology of uninfected wild-type and transgenic, blood neutropenia (absolute neutrophil count 3 in injection of 100 g of RB6-8C5 mAb. This resulted in peripheral neutrophil numbers or function are at highest risk of developing Aspergillus infections, we utilized transiently neutrophil-depleted mice to assess the effect of KC overexpression in this infection. Transient neutrophil depletion was achieved in wild-type and transgenic animals with lung-specific overexpression of KC by i.p. injection of 100 µg of RB6-8C5 mAb. This resulted in peripheral blood neutropenia (absolute neutrophil count <50 cells/µl) on days 1 and 3 after injection in all animals, with restoration of peripheral counts to pretreatment levels by day 5 (data not shown). Animals were challenged with 1–2 × 10^6 i.t. A. fumigatus conidia after 24 h. Lung histology of uninfected wild-type and transgenic animals was normal and was not affected by administration of RB6-8C5 mAb.

In survival studies, KC transgenic animals had a 46% reduction in mortality compared with wild-type animals (Fig. 10A). Lung chitin levels measured 2 days after challenge with conidia showed KC transgenic animals to have a 67% decrease in lung chitin content as compared with wild-type controls (Fig. 10B). Lung histology obtained on day 2 showed a greater accumulation of neutrophils associated with areas of fungal hyphae in KC transgenic mice, as compared with wild-type animals (data not shown).

**Discussion**

Invasive pulmonary aspergillosis is a devastating complication of immunosuppression, which is associated with abnormal numbers or function of neutrophils. ELR^+ CXC chemokines have been shown to have a critical role in neutrophil-mediated events in other diseases. We therefore tested the hypothesis that the interaction of these ligands with CXCR2 is a major component of neutrophil-dependent pulmonary host defense against A. fumigatus, mediating neutrophils deployment in response to the organism.

In order to evaluate the role of neutrophils in host defense against A. fumigatus in an animal model, we developed a model of transient in vivo neutrophil depletion. The Ab-mediated depletion of the available pool of mature neutrophils allowed the organism to establish tissue invasion, which was not observed in animals with intact neutrophil number and function. The dynamics of reduction and early recovery of neutrophil numbers in this model, induced by a single dose of RB6-8C5 mAb on day −1, are analogous to the clinical recovery of neutrophils in patients after chemotherapy or bone marrow transplantation. Two days after i.t. challenge with Aspergillus, the number of neutrophils in the lungs of RB6-8C5-treated animals was larger than that in normal controls challenged with the same inoculum. This greater influx of neutrophils in RB6-8C5-treated animals was in response to fungal tissue invasion and occurred in the context of peripheral blood neutropenia, representing maximal deployment of the limited pool of newly produced neutrophils to the site of infection. An advantage of this model is that it is unaffected by the pleotropic influence of chemotherapeutic agents on various other immunologically active cells. Furthermore, clinical and histological features of invasive aspergillosis in this model resembled those seen in human disease.

![FIGURE 7. Effect of anti-CXCR2 serum on survival in normal mice. Mice were treated with either control or anti-CXCR2 serum i.p. 2 h prior to inoculation with A. fumigatus (2 × 10^7 conidia). *, p < 0.05 as compared with animals receiving control serum. Af, A. fumigatus; anti-CXCR2, anti-CXCR2 serum. Experimental n = 15/group. Data shown are representative of two experiments.](http://www.jimmunol.org/)

![FIGURE 8. Effect of CXCR2 neutralization on lung histopathology after A. fumigatus challenge. A and B, Representative lung H&E and GMS stains in mice treated with control serum, 2 days after inoculation with 1–2 × 10^7 A. fumigatus conidia (magnification, ×400). Inflammatory cellular infiltrate occurred in peribronchial areas, corresponding to site of conidial deposition. Hyphal forms were not present. C and D, Representative lung H&E and GMS stains in mice treated with anti-CXCR2 serum, 2 days after inoculation with 1–2 × 10^6 A. fumigatus conidia (magnification, ×400). Predominantly mononuclear inflammatory cellular infiltrate occurred peribronchially, in areas in which branching hyphae were present.](http://www.jimmunol.org/)
Given that the ELR<sup>+</sup> CXC chemokines have been shown to mediate neutrophil recruitment and activation in diverse conditions, we elected to study their contribution to neutrophil-mediated host defense against <i>A. fumigatus</i>. Other studies have suggested a role for chemokines in host defense against <i>A. fumigatus</i>. For example, isolated rat alveolar macrophages have been shown to produce MIP-1<sub>a</sub>, MIP-2, and KC, as well as TNF-α in response to <i>A. fumigatus</i> conidia in vitro (52), and knockout mice lacking CC chemokine receptor-1 (CCR1), a receptor for MIP-1<sub>a</sub> and RANTES, developed disseminated infection when administered <i>A. fumigatus i.v.</i> (53). In a previous study, we showed that neutralization of TNF resulted in reduced lung neutrophil influx associated with attenuated levels of lung CXC and CC chemokines, including MIP-2, MIP-1α, and JE (54). In this context, we examined the importance of ELR<sup>+</sup> CXC chemokines as a group against <i>A. fumigatus</i>, by blocking their common receptor in the mouse. This approach allowed us to overcome the difficulties in simultaneous neutralization of the biologic effects of multiple ligands, which may have redundant and overlapping functions. The effects of ELR<sup>+</sup> CXC chemokines in humans are mediated via both CXCR-1 and CXCR-2. While the relative simplicity of the murine model allowed us to address the role of these mediators in host defense against <i>A. fumigatus</i>, the dissimilarities between the murine and human systems represent a limitation of the present study. We employed a goat anti-murine serum, which did not deplete circulating neutrophils but prevented in vivo influx of neutrophils into the peritoneum in response to exogenous KC. The pneumonia that resulted from <i>Aspergillus</i> inoculation in the setting of CXCR2 neutralization was indistinguishable from that caused by neutrophil depletion. The residual lung accumulation of neutrophils in the setting of CXCR2 neutralization indicates that other mechanisms, such as other chemotactic factors or differential expression of adhesion molecules, may play a role in neutrophil influx in response to <i>A. fumigatus</i>. However, the magnitude of effect of CXCR2 neutralization highlights the essential role of CXCR2 ligands in host defense against <i>A. fumigatus</i>, nearly to the exclusion of other chemotactic mediators.

The two best-studied murine ELR<sup>+</sup> CXC chemokines, MIP-2 and KC, are functional homologues of the human ELR<sup>+</sup> CXC chemokines, IL-8 and Gro-chemokines (55, 56). MIP-2 and KC...
have been shown to mediate neutrophil-dependent host defense in other models of pneumonia. Specifically, in a murine model of *Pseudomonas* pneumonia, MIP-2 and KC levels were associated with presence of neutrophils in lung (57), and in murine *Klebsiella* pneumonia, MIP-2 was shown to be involved in recruitment of neutrophils, but had only modest effects on clearance of the pathogen and survival of animals (29). In preliminary studies, we found that Ab-mediated depletion of MIP-2 resulted in minimal change in survival in animals challenged with *Aspergillus* (data not shown).

We found markedly greater levels of KC and MIP-2 in the lungs of transiently neutrophil-depleted animals, as compared with immunocompetent animals, challenged with *A. fumigatus*. This difference may be due to the greater burden of organisms in animals with transient neutrophil depletion. Other mechanisms may include local negative feedback on the production of the chemokines, or their receptor-mediated uptake by neutrophils. The present study showed that lung-specific overexpression of KC resulted in augmented host defense against *Aspergillus*, as manifested by reduced mortality and lung fungal burden. Given that immunocompetent mice are intrinsically resistant to *Aspergillus* infection, and that neutropenic patients are at greatest risk of developing invasive aspergillosis, we used a mAb to transiently deplete neutrophils to assess the effect of KC overexpression in the lung. The limited pool of available neutrophils induced in these animals is analogous to the clinical setting in which invasive aspergillosis occurs. The mechanism of the protective effect of KC against *A. fumigatus* is likely via enhanced recruitment of this limited pool of available neutrophils to the lung, as suggested by histology, and may also involve greater neutrophil antifungal activity. These findings are consistent with the prior observation of a protective role for KC in murine bacterial pneumonia, which was associated with an augmented neutrophil influx (58).

The substantial effect of CXCR2 neutralization suggests that multiple ligands may be involved. Other candidate murine ELR\(^+\) CXC chemokines are less well characterized. A newly described murine chemokine, LIX, has been shown to share structural homology with the human chemokines ENA-78 and GCP-2 (18), and is chemotactic for neutrophils in vitro (59). In a murine model of systemic endotoxemia, the expression of the ELR\(^+\) CXC chemokines was found to differ markedly in various organs (19), with chemokines being expressed by lung epithelial cells, but not expressed in other organs. The preferential expression of chemokines in specific organs is of interest, and suggests that the various ELR\(^+\) CXC chemokines may have distinct biological roles.

This study has identified CXCR2 ligands as required components of host defense against *A. fumigatus*. This family of mediators may be important targets in devising future therapeutic strategies against invasive aspergillosis. We have shown that transgenic overexpression of KC in the lungs is feasible and is not complicated by detrimental effects (58). Future studies will evaluate the effect of overexpression or augmentation of other ELR\(^+\) CXC chemokines on the outcome of invasive aspergillosis.

References