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Impaired Pulmonary Host Defense in Mice Lacking Expression of the CXC Chemokine Lungkine

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Lungkine (CXCL15) is a novel CXC chemokine that is highly expressed in the adult mouse lung. To determine the biologic function of Lungkine, we generated Lungkine null mice by targeted gene disruption. These mice did not differ from wild-type mice in their hematocrits or in the relative number of cells in leukocyte populations of peripheral blood or other tissues, including lung and bone marrow. However, Lungkine null mice were more susceptible to Klebsiella pneumoniae infection, with a decreased survival and increased lung bacterial burden compared with infected wild-type mice. Histologic analysis of the lung and assessment of leukocytes in the bronchoalveolar lavage revealed that neutrophil numbers were normal in the lung parenchyma, but reduced in the airspace. The production of other neutrophil chemomtactants in the Lungkine null mice did not differ from that in wild-type mice, and neutrophil migration into other tissues was normal. Taken together, these findings demonstrate that Lungkine is an important mediator of neutrophil migration from the lung parenchyma into the airspace.

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3 Abbreviations used in this paper: ES, embryonic stem; p.i., postinoculation; BAL, bronchoalveolar lavage; MIP-2, macrophage-inflammatory protein-2.
conducted in accordance with the institutional guidelines of Schering-Ingelheim. All experiments in which animals were used were conducted in accordance with the institutional guidelines of Schering-Plough and the University of Michigan.

**RNA analysis**

Total RNA was extracted from lung tissues of wild-type and Lungkine\(^{-/-}\) mice using an ULTRASPEC RNA isolation reagent (Biotex, Houston, TX). Twenty micrograms of RNA was electrophoresed in a 1% agarose gel and blotted onto a Duralon membrane (Stratagene). A Lungkine cDNA probe was \(^{32}\)P-labeled using the Megaprime DNA labeling random primer kit (Amer sham Pharmacia Biotech) according to the manufacturer’s instructions. Hybridization was conducted at 68°C in QuickHyb hybridization solution (Stratagene), and the blots were washed according to the manufacturer’s protocol. The membranes were then exposed overnight to x-ray film at \(-70^\circ\)C with an intensifying screen.

**Histology and immunohistochemistry**

Tissues were either fresh-frozen for cryosection or perfused, inflated (for lung only), fixed in 4% paraformaldehyde, and processed for paraffin sections. Routinely, 5-μm paraffin sections were cut and stained with hematoxylin and eosin. For immunostaining, fresh-frozen sections were fixed with cold acetone and stained with anti-CD11b/Mac-1 or anti-Gr-1 Abs (PharMingen, San Diego, CA). The lung homogenates were placed on ice, and serial 1/5 dilutions were made. Ten microliters of each dilution was plated on soy-base blood agar plates (Difco) and incubated for 18 h at 37°C. Ten microliters of each dilution was plated on soy-base blood agar plates (Difco) and incubated for 18 h at 37°C, and then the colonies were counted.

**Preparation of lung single-cell suspension**

Single-cell suspensions of the lungs were prepared as previously described (8). Briefly, freshly resected lungs were minced with scissors to a fine slurry and incubated at 37°C for 30 min in an RPMI 1640 solution (Sigma, St. Louis, MO) containing 1 mg/ml type A collagenase (Roche, Indianapolis, IN) and 20 U/ml DNase (Sigma). The solutions were then drawn up on ice for 30 min, then centrifuged at 2500 rpm for 10 min. Supernatants were collected, passed through a 0.45-μm pore size filter (Gelman Sciences, Rutherford, NJ). Hematologic values were determined with an automated hematologic analyzer (Hematology, Vacutainer Systems; Becton Dickinson, Becton Dickinson, NJ). Blood samples were collected from the infraorbital sinus into sterile evacuated tubes with added EDTA (Vacutainer Systems; Becton Dickinson, Rutherford, NJ). Hematologic values were determined with an automated system (Cell-Dyn 3500; Abbott, Chicago, IL). Platelet counts were performed manually when the instrument was unable to provide accurate platelet counts due to excessive clumping or excessively large platelets.

**Bronchoalveolar lavage (BAL)**

BAL was performed to obtain airspace cells as previously described (11). The trachea was exposed and intubated using a 0.97-mm outside diameter polyethylene catheter. BAL was performed by instilling PBS containing 5 mM EDTA in 1-ml aliquots. Approximately 5 ml of lavage fluid was retrieved per mouse. Cytospins were then prepared from BAL cells and stained with Diff-Quik (Dade Behring, Newark, DE), and differential counts were determined.

**Lung harvesting for cytokine analysis**

At designated time points, lungs were harvested and placed in 3 ml of sterile saline, and the tissues were homogenized with a tissue homogenizer under a vented hood. The lung homogenates were plated on ice, and serial 1/5 dilutions were made. Ten microliters of each dilution was plated on soy-base blood agar plates (Difco) and incubated for 18 h at 37°C, and then the colonies were counted.

**Murine cytokine ELISA**

Murine TNF-α, macrophage-inflammatory protein-2 (MIP-2), and KC were measured using a murine cytokine ELISA (R&D Systems, Minneapolis, MN) as described previously (11, 12). Briefly, flat-bottom 96-well microtiter plates (Immunoplate II 96-F; Nunc, Copenhagen, Denmark) were coated with 50 μl/well rabbit Ab against the various cytokines (1 μg/ml in 0.6 M NaCl, 0.2 M H3BO3, and 0.08 M NaOH, pH 9.6) for 16 h at 4°C, then washed with PBS (pH 7.5) and 0.05% Tween 20 (wash buffer). Non-specific binding sites on the microtiter plates were blocked with 2% BSA in PBS and incubated for 90 min at 37°C. After rinsing four times with wash buffer, the plates were incubated with diluted (undiluted and 1/10) cell-free supernatants (50 μl) in duplicate, followed by incubation for 1 h at 37°C. Plates were washed four times, followed by the addition of 50 μl/well biotinylated rabbit Ab against the specific cytokines (3.5 μg/ml in PBS

**Histology and immunohistochemistry**

For the experiments described here we used Lungkine\(^{-/-}\) mice generated by interbreeding these heterozygous mice. Stained samples were kept in the dark at 4°C until analyzed.

**Flow cytometry**

Total lung leukocytes were isolated from animals as described above. For identification of lung leukocyte subsets, FITC- or PE-conjugated CD4, CD8a, CD3, CD11c, B220, pan NK, Mac-1, F4/80, and Gr-1 were used (all reagents from PharMingen). In addition, cells were stained with anti-CD45 (PharMingen), allowing discrimination of leukocytes from nonleukocytes and thus eliminating any nonspecific binding of cell surface markers on nonleukocytes. Stained samples were kept in the dark at 4°C until analyzed on a FACSScan cytometer (Becton Dickinson, San Jose, CA) using CellQuest software (Becton Dickinson). Leukocyte populations were analyzed by gating on CD45-positive cells of the appropriate light scatter characteristics and then were examined for FL1 and FL2 fluorescence expression. The absolute number of a leukocyte type was determined by multiplication of the percentage of that cell type by the total number of lung leukocytes in that sample. For analyses of bone marrow and blood, single-cell suspensions were prepared from individual tissues by passage through a 100-μm pore size nylon cell strainer (BD Biosciences Labware, Bedford, MA) in RPMI 1640 medium containing 10% FCS.

**Hematology**

Blood samples were collected from the intra-abdominal sinus into sterile evacuated tubes with added EDTA (Vacutainer Systems; Becton Dickinson, Rutherford, NJ). Hematologic values were determined with an automated system (Cell-Dyn 3500; Abbott, Chicago, IL). Platelet counts were performed manually when the instrument was unable to provide accurate platelet counts due to excessive clumping or excessively large platelets.

**Klebsiella pneumoniae inoculation**

We chose to use K. pneumoniae strain 43816, serotype 2 (American Type Culture Collection, Manassas, VA) in our studies because a murine model of pneumonia has been well characterized using this strain (9, 10). K. pneumoniae was grown in tryptic soy broth (Difco, Detroit, MI) for 18 h at 37°C. The concentration of bacteria in broth was determined by measuring the amount of absorbance at 600 nm. A standard of absorbencies based on known CFU was used to calculate the inoculum concentration. An inoculum of 1 \times 10^7 organisms was chosen, as this dose allowed for the development of substantial inflammation by 36–48 h without excessive mortality in wild-type animals. Mice were anesthetized with 1.8–2 mg of pentobarbital/animal i.p. The trachea was exposed, and 30 μl of inoculum or saline was administered via a sterile 26-gauge needle. The skin incision was closed with surgical staples.

**Determination of lung K. pneumoniae CFU**

At the time of sacrifice, the right ventricle was perfused with 1 ml of PBS, the lungs were removed aseptically and placed in 3 ml of sterile saline, and the tissues were homogenized with a tissue homogenizer under a vented hood. The lung homogenates were plated on ice, and serial 1/5 dilutions were made. Ten microliters of each dilution was plated on soy-base blood agar plates (Difco) and incubated for 18 h at 37°C, and then the colonies were counted.

**Bronchoalveolar lavage (BAL)**

**Lung harvesting for cytokine analysis**

At designated time points, lungs were harvested and placed in 3 ml of sterile saline, and the tissues were homogenized with a tissue homogenizer under a vented hood. The lung homogenates were plated on ice, and serial 1/5 dilutions were made. Ten microliters of each dilution was plated on soy-base blood agar plates (Difco) and incubated for 18 h at 37°C, and then the colonies were counted.
(pH 7.5), 0.05% Tween 20, and 2% FCS), and incubated for 30 min at 37°C. Streptavidin-peroxidase conjugate (Bio-Rad, Richmond, CA) was then added after washing the plates, and incubation proceeded 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 ml/well 3 M H2SO4 solution. Plates were read at 490 nm in an ELISA plate reader. Standards were half-log dilutions of recombinant murine cytokines from 1 pg/ml to 100 ng/ml. This ELISA method consistently detected murine cytokine concentrations >25 pg/ml. The ELISA did not cross-react with IL-1, IL-2, IL-4, or IL-6. In addition, the ELISA did not cross-react with other members of the murine chemokine family, including murine MIP-1, JE, RANTES, or ENA-78.

**In vivo neutrophil chemotaxis**

The mouse air-pouch model for in vivo chemotaxis has been described in detail previously (13). Briefly, sex- and age-matched Lungkine−/− and wild-type mice were anesthetized with ether. On experimental day 0, 5 ml of sterile air was injected s.c. under the dorsal skin; the resultant space was reinjected with 3 ml of sterile air on day 3. On day 5, 0.2 μg of Escherichia coli LPS (Sigma) in 1 ml of carboxymethylcellulose (0.5% in saline; Fluka, Buchs, Switzerland) was injected into the pouches. The animals were sacrificed 4 h later, and the air-pouches were lavaged with 2 ml of sterile PBS. The resulting cell suspensions were pelleted, resuspended, and counted under a hemocytometer. Cytospin slides were prepared and stained (Diff-Quik Stain set; Dade Behring), and differential cell counts were determined using a high-power microscope. The absolute number of a leukocyte type was determined by multiplication of the percentage of that cell type by the total number of lung leukocytes in that sample.

**Statistical analysis**

Data were analyzed using the InStat version 2.01 statistical package (GraphPad software; GraphPad, San Diego, CA). Survival data were compared using Fisher’s exact test. All other data were expressed as the mean ± SEM and compared using an unpaired two-tailed Mann-Whitney test.
mRNA expression in the lung tissues of Lungkine−/− mice is not essential for embryonic development. We expected Mendelian ratios, demonstrating that Lungkine expression is not essential for embryonic development.

Leukocyte populations in the lung were analyzed by flow cytometry. We did not detect significant differences in the absolute or relative numbers of distinct leukocyte populations (CD3-, CD4-, CD8a-, CD11c-, B220-, pan NK-, Mac-1-, F4/80-, Gr-1-, CD4-, CD8a-, and CD3-expressing subsets; n = 14 for bone marrow and n = 10 for blood; data not shown). Similarly, hematocrits and total white blood cell counts of Lungkine−/− mice did not differ significantly from those of wild-type mice (n = 15; data not shown).

Lungkine−/− mice have normal leukocyte subpopulations

To evaluate a possible role for Lungkine in hemopoiesis, leukocyte subpopulations in peripheral blood and bone marrow were analyzed by flow cytometry. Compared with wild-type mice, Lungkine−/− mice had no significant changes in the absolute or relative numbers of distinct cell types (CD11c-, B220-, pan NK-, Mac-1-, F4/80-, Gr-1-, CD4-, CD8a-, and CD3-expressing subsets; n = 14 for bone marrow and n = 10 for blood; data not shown).

Results

Generation of Lungkine−/− mice

DNA sequence analysis of the Lungkine genomic locus revealed that it contains three introns and four exons. The translation start site is located in exon 1, and the open reading frame spans all four exons (Fig. 1A). A targeting strategy was designed to delete a 3-kb sequence of Lungkine genomic DNA that includes the entire first and second exons and part of the third exon (Fig. 1A). The targeting vector shown in Fig. 1 was used to transfet ES cells. Two independent ES cell clones containing the targeted loci were identified by Southern blot analysis and injected into mouse blastocysts. Chimeras obtained from both clones were bred with C57Bl/6J mice to generate heterozygotes (Lungkine+/−). PCR analysis of tail DNA from these founders confirmed germine transmission of the targeted allele (Fig. 1B). Further intercrossing of these Lungkine+/− mice yielded Lungkine−/− mice within the expected Mendelian ratios, demonstrating that Lungkine expression is not essential for embryonic development.

Northern blot analysis was used next to examine Lungkine mRNA expression in the lung tissues of Lungkine−/− mice. As shown in Fig. 1C, a Lungkine mRNA of the predicted size was detected in the wild-type mouse, but not in either line of Lungkine−/− mice.

Lungkine−/− mice develop normally and have a normal complement of leukocytes in the lung

The Lungkine−/− mice developed normally and were fertile. Routine histologic analysis of all organs was unremarkable. Given Lungkine’s expression pattern and its chemotactic properties, we focused our initial analysis on the lung using immunohistochemistry and flow cytometry. Anti-Mac-1 and anti-Gr-1 Abs were used in immunohistochemical staining to examine the resident macrophage and neutrophil populations in the lung tissues of Lungkine−/− animals. No significant differences from wild-type mice were seen in the number or localization of these cell types in the lungs of Lungkine−/− mice (data not shown). The resident leukocyte population in lung tissue was further analyzed by flow cytometry. We did not detect significant differences in the absolute or relative numbers of distinct leukocytes (CD3-, CD4-, CD8a-, CD11c-, B220-, pan NK-, Mac-1-, F4/80-, and Gr-1-expressing subsets) between Lungkine−/− and wild-type animals (n = 10; data not shown).

Lungkine−/− mice are more susceptible to pneumonia induced by Klebsiella

Given the known chemotactic properties of this molecule for neutrophils and its high levels of expression in the lung (5), we next examined the susceptibility of Lungkine−/− mice to Klebsiella pneumonia. Both Lungkine−/− and wild-type animals developed lethargy and ruffled fur 48 h postinoculation (p.i.) with a sublethal dose. However, only 56% of Lungkine−/− mice survived for longer than 10 days compared with an 85% survival rate for wild-type mice (Fig. 2).

To investigate the cause of the observed increased mortality in Lungkine−/− mice, we measured the lung bacterial burden of Lungkine−/− and wild-type control mice. Lungs of animals challenged with 1 × 10⁵ CFU K. pneumoniae were harvested 48 h p.i. As shown in Fig. 3, there was a 73-fold increase in lung bacterial burden in Lungkine−/− mice compared with wild-type controls. This observation indicated that the absence of Lungkine resulted in significant impairment of the clearance of K. pneumoniae from the lung.

Characterization of inflammatory cell influx in lungs of K. pneumoniae-infected mice

To determine whether the increased bacterial CFU in Lungkine−/− mice was associated with impaired cellular infiltration into the lung, we first examined the lungs of infected mice by histologic analysis at 24 and 48 h following Klebsiella inoculation. These time points were chosen to examine both early and maximum influxes of leukocytes, respectively (9). As shown in Fig. 4, a dense neutrophilic infiltrate was observed in both the interstitial and alveolar compartments of wild-type lung. In contrast, neutrophils were strikingly absent from the alveoli of Lungkine−/− lung sections at 24 h p.i. In addition, compared with wild-type mice, an
increase in basophilically stained bacterial particles was seen in lungs of Lungkine−/− mice at 48 h p.i. (Fig. 4, C and D). To further characterize the apparent absence of airspace neutrophils in Lungkine−/− mice, we quantified the cell numbers in the BAL. In uninfected mice no differences in the relative or absolute numbers of total BAL cells, neutrophils, or mononuclear cells were noted between wild-type and Lungkine−/− mice. However, at 24 h p.i., an average 10-fold increase in the numbers of BAL neutrophils was observed in wild-type mice compared with baseline, whereas there was no appreciable increase in the numbers of airspace neutrophils in Lungkine−/− mice (Fig. 5A). In contrast, at 48 h the numbers of BAL neutrophils in Lungkine−/− mice was even greater than that in wild-type animals, although this difference was not significant (p = 0.14). No significant differences in numbers of BAL mononuclear cells were observed at either 24 or 48 h following Klebsiella challenge (Fig. 5B).

Lungkine is produced primarily by airway epithelial cells and is secreted into the airway (5). To determine whether Lungkine preferentially affects infiltration of neutrophils into the airway or into the parenchyma, we infected mice with K. pneumoniae and prepared cells from both the lung airspace (BAL) and the parenchyma 24 h after bacterial challenge. As shown above, a reduction in the numbers of infiltrating neutrophils was observed in the airspace compartment of Lungkine−/− mice compared with that in wild-type mice. In contrast, higher numbers of neutrophils were observed in the parenchyma of Lungkine−/− mice compared with that of wild-type mice, although no statistically significant difference was seen between the two groups (Fig. 6). This result suggests that a major function of Lungkine is to facilitate migration of neutrophils from the parenchyma into the airway.

Analysis of lung neutrophil-chemotactic mediators and extrapulmonary neutrophil chemotaxis in Lungkine−/− mice

We next addressed whether a reduction in airway neutrophils of Lungkine-deficient mice is secondary to impaired production of other neutrophil chemotactic mediators. Levels of TNF-α, MIP-2, and KC in the lung were measured 24 h p.i. with K. pneumoniae. No significant differences in the levels of these cytokines were
seen between lungs of wild-type and Lungkine-deficient animals (data not shown), indicating that the reduced neutrophil influx into the airway of Lungkine−/− mice is independent of other neutrophil chemoattractants.

To assess whether the Lungkine deficiency results in a general defect in neutrophil mobilization, we measured neutrophil chemotaxis at an extrapulmonary site. We used a well-characterized model in which LPS is injected into an s.c. air-pouch, (13, 14). As shown in Fig. 7, at 4 h postinjection, an early time point at which the accumulation of neutrophils can be reliably assessed (14), there was no significant difference in the number of neutrophils between wild-type and Lungkine−/− animals (1.03 × 10^7 ± 2.9 × 10^6 and 8.6 × 10^6 ± 2.7 × 10^6 neutrophils, respectively). This indicates that Lungkine is not generally required for neutrophil trafficking and suggests that it is specific to the airspace of the lung.

**FIGURE 6.** Parenchymal neutrophil cell numbers in the lungs of wild-type and Lungkine−/− mice 24 h after K. pneumoniae inoculation. Data represent the average values of two separate experiments. There were 12 mice/group. p = 0.29 compared with wild-type mice challenged with K. pneumoniae.

**FIGURE 7.** Number of neutrophils in the s.c. air-pouch in wild-type and Lungkine−/− mice after LPS instillation. LPS was injected into the air-pouches in wild-type and Lungkine−/− mice, and the infiltrating cells were lavaged at 4 h after injection as described in Materials and Methods. There were six mice per group. p = 0.65 compared with wild-type mice challenged with LPS.

**Discussion**

In this report, we describe the generation and preliminary characterization of mice lacking the novel CXC chemokine Lungkine. In the adult mouse, Lungkine is produced at appreciable levels only by lung epithelial cells and collects in the lung airspace, suggesting that it might function in pulmonary host defense. We now show that deletion of the Lungkine gene is associated with diminished host defense against the pulmonary pathogen K. pneumoniae.

The rapid clearance of bacterial pathogens from the respiratory tract is mediated by resident alveolar macrophages and neutrophils that are recruited from the blood into the airspace (15–17). This neutrophil recruitment is mediated by the production in the lung of chemotactic cytokines (16). ELR⁺ CXC chemokines, including MIP-2 and KC, contribute to antibacterial host defense by affecting neutrophil trafficking and activation (9, 11, 18).

The increased mortality in Lungkine−/− mice following infection with K. pneumonia demonstrates that Lungkine is a chemokine that has a central role in pulmonary host defense. Our finding that the numbers of neutrophils are decreased in the BAL of infected Lungkine−/− mice at 24 h p.i. (but not in the parenchyma) suggests that one function of Lungkine might be to direct neutrophils from the parenchyma into the airspace. This reduction may be of notable functional importance, given the key role of neutrophils in innate defense against bacteria in the respiratory tract that have not been killed by resident alveolar macrophages (15). In fact, at 48 h p.i., the burden of bacteria was considerably greater in Lungkine−/− mice than in wild-type control animals. The increased bacterial load may lead to tissue invasion and dissemination that is not adequately controlled despite the impressive influx of neutrophils seen in Lungkine−/− mice at this time. A similar increase in both neutrophils and bacteria was seen in mice lacking the receptor for neutrophil chemottractant C5a following infection with Pseudomonas aeruginosa, although the C5a-deficient mice did not display the early deficit in airway neutrophils seen in the Lungkine−/− mice (19). This increased neutrophilic content found in the airways of both these mouse strains is probably secondary to an increased bacterial load. However, as in all genetically engineered, loss-of-function mice, we cannot exclude the possibility that other unidentified, compensatory neutrophil attractants are up-regulated in Lungkine−/− mice. Lungkine protein has been shown by others to be secreted into the alveolar space in response to inflammatory stimuli (5). This pattern of expression and secretion may favor directed migration of leukocytes from the vascular and/or interstitial spaces into the airway lumen and alveolar spaces. If that is the case, it is unclear why so few neutrophils are normally present within the lung airspace and airway despite the fact that Lungkine is expressed at high levels in these structures. Apparent paradoxes of this sort are not unique to Lungkine. Other chemokines, such as eotaxin (20, 21), have potent chemotactic roles in vitro and are expressed at high levels in tissues that are not normally infiltrated by eosinophils, the target cells of eotaxin. We propose that Lungkine may act as a permissive factor, facilitating the transmigration of neutrophils into the airways during the early phases of inflammation. For example, Lungkine may act specifically on activated neutrophils or may work in concert with undefined molecules that are present only on the surface of inflamed endothelium. It is also possible that Lungkine, like other ELR⁺ CXC chemokines, may affect polymorphonuclear leukocyte activities, including respiratory burst and antimicrobial activities (1, 2, 22). Finally, the site of production (distal airway epithelial cells) and the magnitude of expression show similarities to the expression of defensin proteins (23), and this raises the possibility that Lungkine may have direct antimicrobial properties that are
independent of its effects on neutrophils. Exploring these mecha-
nistic possibilities will probably contribute to a better under-
standing of the role of Lungkine in host defenses and to insights into the
biology of a subset of chemokines expressed constitutively in spe-
cific organs.

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