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Lungkine, a Novel CXC Chemokine, Specifically Expressed by Lung Bronchoepithelial Cells[1]

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We describe a novel mouse CXC chemokine that is selectively expressed in lung epithelial cells and up-regulated in various lung inflammation models. Although this chemokine clusters with other ELR-CXC chemokines, none of them can confidently be assigned to be its human homologue based on sequence identity. In addition, the highly restricted mRNA tissue distribution of this chemokine differentiates it from all previously described chemokines: Lungkine could not be detected in any of the 70 cDNA libraries analyzed corresponding to specific murine cell populations and tissues. High levels of Lungkine mRNA were specifically detected in the lung and at lower levels in fetal lung tissue by Northern blot and in situ hybridization, suggesting a potential role for this chemokine during lung development. Moreover, Lungkine protein is secreted into the airway spaces and induces the in vitro and in vivo migration of neutrophils, suggesting that it is involved in lung-specific neutrophil trafficking. Using fluorescent in situ hybridization, we show that Lungkine maps to mouse chromosome 5. *The Journal of Immunology*, **1999, 162: 5490–5497.**

Chemokines are basic heparin-binding proteins that, in association with adhesion molecules, play a pivotal role in selectively recruiting certain subsets of leukocytes to specific sites of inflammation and tissue injury (1–3).

Many chemokines are expressed constitutively and/or during inflammation in the lung as well as in other tissues. Examples include epithelial cell-derived neutrophil activating protein-78 (ENA-78) (4, 5), eotaxin (6), macrophage inflammatory protein-3α (MIP-3α)[3] (7), RANTES (8), IL-8, (9) and many others.

The redundancy in chemokine production by resident and/or infiltrating cells in the lung may reflect the need for rapid cell recruitment in response to the large number of Ags that penetrate the lungs.

During the last few years, one of our goals has been to identify and characterize new chemokines. We initially reported human MIP-3α (7, 10, 11), which was recognized as a chemokine that is abundantly produced in several organs (7). While trying to isolate a cDNA clone encoding mouse MIP-3α by screening lung cDNA libraries, we unexpectedly found a new mouse CXC chemokine that we have designated Lungkine.

Lungkine differs from all previously described chemokines because its expression is restricted to lung bronchoepithelial cells and is up-regulated during inflammation. The subfamily of the ELR-CXC chemokines is known to induce the migration of neutrophils and, in some cases, T cells, although the latter is controversial (12, 13). Acute inflammation is characterized by a prevalent neutrophilic infiltrate, which are the first leukocytes to enter a site of allergic inflammation. For this reason, we studied the regulation of Lungkine mRNA expression in normal or in various murine inflamed lung tissues, including lungs from OVA- and *Aspergillus*-challenged mice, which are recognized asthma models. Our data suggest that this new lung-specific chemokine may play a role in neutrophil trafficking during normal and inflammatory conditions.

Materials and Methods

Isolation of murine Lungkine cDNA

A total of 150,000 clones from a RAG-1$^{-/-}$ lung mouse cDNA plasmid library were screened using a human MIP-3α cDNA probe (7). The hybridization was conducted in Churches solution at 65°C (14), and filters were washed at medium stringency (1× SSC/0.1% SDS at 65°C). One positive clone (clone 20) was isolated and sequenced following the second round of screening. The new cDNA clone was used as a probe in the screening of a *Nippostrongylus brasiliensis*-infected mouse lung cDNA library to obtain the full-length Lungkine cDNA. Three positive clones were obtained after screening 100,000 clones. Most of the work described here was done with the full-length clone N4C. The nucleotide sequences of Lungkine cDNA clones were confirmed by both-strands automated sequencing using an Applied Biosystems 373 sequencer (Foster City, CA). All sequences obtained were imported into and analyzed using Sequencher (Genecodes, Ann Harbor, MI).

Murine Lungkine mRNA distribution

Total RNA was made from different tissue sources and cell lines using RNAzol B solution (Tel-Test, Friendswood, TX). A total of 15 μg of RNA was loaded in each lane, transferred to a Hybond-N membrane (Amersham, Arlington Heights, IL), hybridized, and washed at high stringency (0.2× SSC/0.1% SDS at 65°C). A mouse tissue Northern blot from Clontech (Palo Alto, CA) was hybridized to confirm mRNA distribution. Lungkine cDNA (1 kb) and a 0.4-kb cDNA fragment of hypoxanthine phosphoribosyltransferase (HPRT) were used as probes in all Northern blot analyses.

Southern blot analysis of cDNA libraries

A panel of mouse cDNA libraries was analyzed by Southern blot as described previously (15).

Chromosomal mapping

The 1-kb Lungkine cDNA probe was used by Genome Systems (St. Louis, MO) to determine the chromosomal localization by fluorescent in situ hybridization (ISH). The cDNA probe was labeled with digoxigenin dUTP by nick translation. Labeled probe was combined with sheared mouse DNA and hybridized to normal metaphase chromosomes derived from mouse

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[3] Abbreviations used in this paper: MIP, macrophage inflammatory protein; KC, keratinocyte-derived chemokine; cRNA, complementary RNA; HPRT, hypoxanthine phosphoribosyltransferase; ISH, in situ hybridization; BAL, bronchoalveolar lavage; MCP, monocyte chemoattractant protein; CXCR, CXC chemokine receptor ORF, open reading frame; UTR, untranslated region; EST, expressed sequence tag; ENA, epithelial cell-derived neutrophil activating protein.

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embryo fibroblast cells in a solution containing 50% formamide, 10% dextran sulfate, and 2× SSC. Specific hybridization signals were detected by incubating the hybridized slides in fluoresceinated antidigoxigenin Abs followed by counterstaining with 4′,6-diamidino-2-phenylindole.

Immunohistochemistry

Lungs from normal mice and OVA-sensitized and -challenged mice were treated as described previously (16). Sections were incubated with a rabbit polyclonal affinity-purified Ab raised against the Lungkine peptide CLDPDAPWVKATVGPITNRFLPEDLKQKE-COOH (Genemed, South San Francisco, CA). The negative controls used in this experiment were sections incubated in the absence of primary Ab or incubated with a blocking mix of peptide and primary Ab. The peptide was used in a 10 molar excess in respect to the Ab to ensure blocking.

In situ hybridization

ISH was performed as described previously (17). Briefly, fetuses and tissues were fixed in 4% paraformaldehyde in PBS overnight, dehydrated, and infiltrated with paraffin. Serial sections at a thickness of 5–7 μ were remounted on gelatinized slides. Sections were deparaffinized in xylene, rehydrated, and postfixed. The sections were digested with proteinase K, postfixed, treated with triethanolamine/acetic anhydride, washed, and dehydrated. Complementary RNA (cRNA) was prepared from linearized cDNA templates to generate antisense and sense probes. The cRNA transcripts were synthesized according to the manufacturer's instructions (Ambion, Austin, TX) and labeled with [^{35}S]UTP (>1000 Ci/mmol; Amersham). cRNA transcripts of >200 nucleotides were subjected to alkali hydrolysis to give a mean size of 70 bases. Sections were hybridized overnight at 52°C in 50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM $NaPO_4$, dextran sulfate, 1× Denhardt's solution, 50 μg/ml total yeast RNA, and 50,000-75,000 cpm/μl ^{35}S-labeled cRNA probe. The tissue was subjected to stringent washing at 65°C in 50% formamide, 2× SSC, and 10 mM DTT and washed in PBS before treatment with 20 μg/ml RNase A at 37°C for 30 min. Following washes in 2× SSC and 0.1× SSC for 10 min at 37°C, the slides were dehydrated, dipped in Kodak NTB-2 nuclear track emulsion, and exposed for 2–3 wk in light-tight boxes with desiccant at 4°C. Photographic development was conducted in Kodak D-19. Slides were counterstained lightly with toluidine blue and analyzed using both the light and dark field optics of a Zeiss Axiophot microscope. Sense control cRNA probes (identical with the mRNAs) indicate the background levels of the hybridization signal.

Recombinant protein expression and purification

Escherichia coli (strain SG220014) was transformed with pMBD101012 plasmid carrying Lungkine cDNA. Cells were grown in freshly prepared 2× Luria-Bertani medium, induced at time 0, and grown at 37°C until the A560 was 2.5. Next, cells were harvested by centrifugation, and the inclusion bodies were isolated by fluidizing the cells in 50 mM Tris buffer (pH 8.5), 5 mM EDTA, and 1 mM Pefa Bloc. The inclusion bodies were washed in 50 mM Tris buffer (pH 8.5) and 5 mM EDTA containing 1% Triton X-100 and subsequently in Tris buffer containing 1 M guanidine HCl. The washed inclusion bodies were solubilized in 50 mM Tris buffer (pH 8.5) containing 8 M guanidine HCL, 5 mM EDTA, 10 mM DTT, and 1 mM Pefa Bloc. The solubilized inclusion bodies were renatured by dilution (100×) in 50 mM Tris (pH 8.5) containing 1.5 mM glutathione, 0.5 mM glutathione disulfide, 0.4 M guanidine, and 5 mM EDTA. The protein was allowed to renature for 16 h and subsequently concentrated and diafiltered into 50 mM Tris (pH 8.5) and 5 mM EDTA. The refolded protein was purified by chromatography on a Baker C4 reverse phase column. The fractions containing Lungkine were recognized by gel electrophoresis and confirmed by Western blot analysis using an anti-peptide Ab. The Lungkine-containing samples were pooled and run over the same Baker C4 column again to remove endotoxin (final concentration of 2 endotoxin units/ml). Final gel analysis demonstrated a single band by gel electrophoresis.

Bronchoalveolar lavage (BAL) and OVA treatment

OVA/alum was prepared as described previously (18). Briefly, alum precipitate was prepared by the addition of 10N NaOH (Sigma, St. Louis, MO) to a 10% solution of $AlKSO_4$ (Sigma) until precipitate formed. This precipitate was then extensively washed with sterile PBS and stored at 4°C. On the day of the injections, OVA (grade V; Sigma) in PBS was bound to the alum precipitate (OVA/alum) by mixing at 4°C. Mice were primed i.p. with 10 μg OVA/alum on day 0 and boosted on day 7. On day 14, mice were aerosolized with OVA in PBS using a Passport aerosol compressor (Invacare, Elyria, OH) connected to a 3-ft^3 box for 20 min. Previous studies have shown that the aerosolization delivers 3–5 μg of OVA to each mouse (18).

Mice were sacrificed at the indicated timepoints following OVA aerosolization, and BALs were performed by intratracheal insertion of a needle. The BAL was harvested in 3 ml of incomplete RPMI 1640 and stored frozen until analysis. BALs were concentrated by precipitating proteins with cold acetone for 1 h at −20°C. The samples were centrifuged, and the pellets were resuspended in PBS. Protein concentration was determined by the Bradford method using Bio-Rad solutions (Bio-Rad, Richmond, CA).

N. brasiliensis *and* Aspergillus *treatment*

BALB/c mice were given 500 *N. brasiliensis* worms in 50 μl of PBS s.c. in the flank on day 0. Lungs were harvested on days 8 and 10, and total RNA was isolated for Northern blot analysis.

BALB/c mice were treated with 50 μg of *Aspergillus* extract in 50 μl of PBS intranasally on day 0, and lungs were harvested 6 h after Ag challenge. Total RNA was prepared and analyzed by Northern blot.

Western blotting

A total of 10 μg of protein was loaded in 18% acrylamide denaturing gels (Novex, San Diego, CA). The procedure was performed following conventional methods (14). Rabbit polyclonal affinity-purified antiserum (Genemed, South San Francisco, CA) was used as the primary Ab for Lungkine detection. Anti-rabbit IgG, HRP, was used as secondary Ab; Super Signal (Pierce, Rockford, IL) solutions were used to develop the blot.

Cell isolation

Thioglycolate medium (1 ml) (Difco Laboratories, Detroit, MI) was injected i.p. in 6- to 8-wk-old BALB/c mice. Mice were sacrificed at 24 h postinjection, and peritoneal lavages were performed with 5 ml of cold PBS. The cells obtained were treated with RBC lysing buffer (Sigma) and counted.

Transwell chemotaxis assay

Chemotaxis was performed as described previously (19). Briefly, the cells and chemokines used in this assay were resuspended in DMEM containing 1% low endotoxin BSA (Sigma) at pH 6.95. Transwell plates of 3-μm pore size (Corning Costar, Cambridge, MA) were coated with Sigmacote (Sigma) and loaded with 600 μl of medium or with different chemokine dilutions in duplicate (lower chamber). Cells were resuspended at 2×10^7 cells/ml, and 100 μl of this suspension was placed in the inserts (upper chamber). After 2 h of incubation at 37°C and 5% CO_2, 50 μl of 70 mM EDTA was added in the lower chamber to release adherent cells from the membrane and the bottom of the plate. Inserts were removed, and 10,000 beads (Dynospheres Uniform Microspheres, mean diameter of 15 μm; Bangs Laboratories, Fishers, IN) were added per well. The cells in the lower chamber were collected along with the starting cell population, stained with Gr-1-FITC (stains granulocytes) (PharMingen, San Diego, CA) and F4/80-PE (stains macrophages) (Caltag, Burlingame, CA) and analyzed by flow cytometry in a FACScan (Becton Dickinson, Milpitas, CA). A total of 10,000 events were collected per analysis. The ratio of beads to cells was determined, allowing us to calculate the number of cells that had migrated to the bottom well.

In vivo chemotaxis

A total of 10 μg of purified recombinant protein, either murine Lungkine or murine keratinocyte-derived-chemokine (KC) (R&D Systems, Minneapolis, MN), LPS (endotoxin-matched control), or PBS (negative control), was injected i.p. into 8-wk-old C3H/HeJ and BALB/C mice. Mice were sacrificed at 3 and 24 h postinjection. Cold endotoxin-free PBS (5 ml) was used to wash the peritoneum and collect cells. The recovered cells were counted. A total of 500,000 cells from each mouse were stained with Gr-1- (PharMingen), F4/80-, and $CD4^+$- and $CD8^+$- (Caltag) conjugated Abs and analyzed by flow cytometry as described above. Results are expressed as the percentage of a specific cell population with respect to the number of total cells counted using a Coulter counter (Coulter, Hialeah, FL).

Results

Cloning and characterization of murine Lungkine cDNA

We initially sought to isolate the mouse homologue of MIP-3α. To achieve this, we analyzed several cDNA libraries by Southern blot with human MIP-3α; a RAG-$1^{-/-}$ lung cDNA library yielded a strong signal. This cDNA library was screened with a human

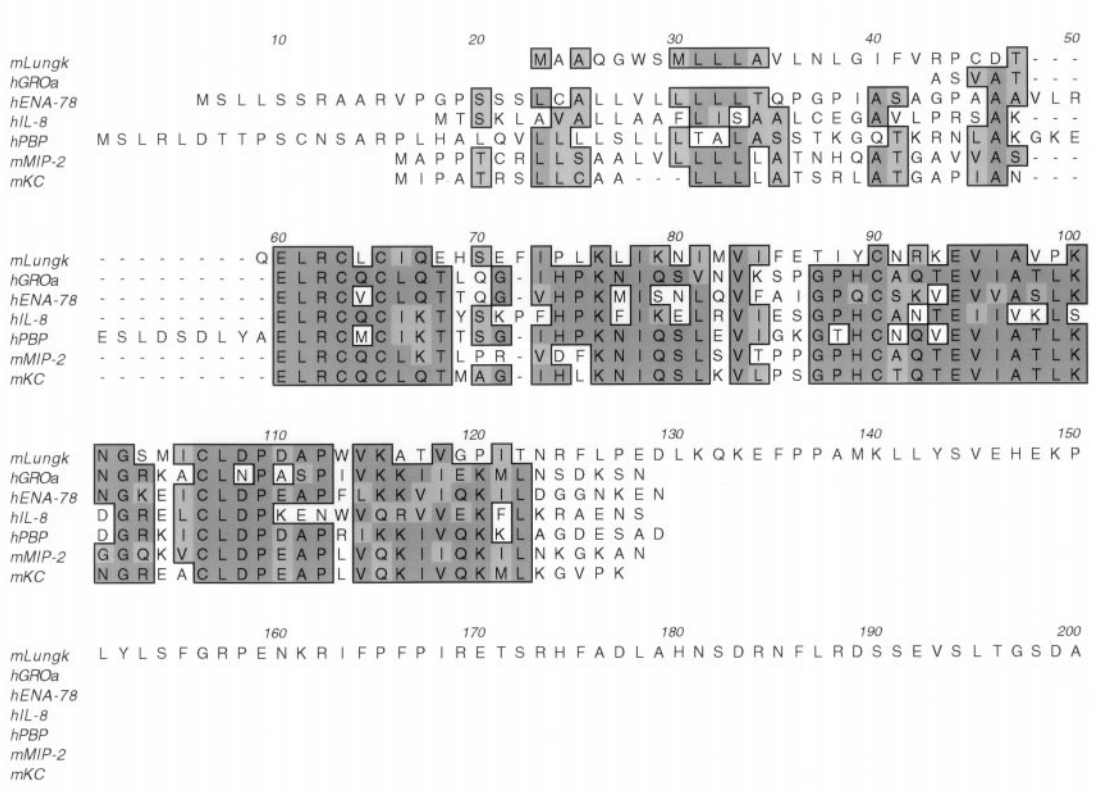

FIGURE 1. Protein sequence comparison. The chemokine protein sequences were aligned using the ClustalW function of Mac Vector 6.0.1. The aligned sequences were chosen based on BLAST searches performed with the mouse Lungkine sequence. Conserved regions between sequences are shown within boxes. (This sequence has been deposited in the GenBank database and assigned accession no. AF082859).

MIP-3α probe. Several positive clones were isolated and sequenced. Unexpectedly, one 1-kb positive clone comprised most of the complete open reading frame (ORF) and the 3′ untranslated region (UTR) sequence of a new ELR-CXC chemokine that had not been described before. At that time, no human or mouse expressed sequence tag (EST) encoding Lungkine was present in the GenBank database of ESTs (dbEST).

A panel of cDNA libraries was analyzed by Southern blot using the 1-kb Lungkine cDNA probe. The libraries that gave a positive signal were: mouse RAG $1^{-/-}$ lung, mouse normal lung, and *N. brasiliensis*-infected lung cDNA libraries (data not shown). The highest abundance of Lungkine cDNA was detected in the *N. brasiliensis*-infected lung cDNA library. Isolation of the full-length cDNA was conducted by screening the latter library. One of the positive clones obtained in the screening, which was ~1 kb in size, contained a 5′UTR sequence, the complete ORF, and the 3′UTR sequence. (This sequence has been deposited in the GenBank database and assigned accession no. AF082859). In addition, three mRNA instability sequences (ATTTA) were present in the 3′UTR, characteristic of cytokine mRNAs (data not shown).

Murine Lungkine cDNA displays a 166-aa ORF (Fig. 1). The processing of a predicted 25-aa leader sequence (http://www.cbs.dtu.dk/services/SignalP/) results in a mature protein of 141 aa with an unusually short N terminus and an extremely long C-terminal tail that protrudes beyond the chemokine fold. The function of this extended tail is unknown; however, it has been shown (20) that severe truncations of the long C terminus of Lymphotactin abolish its chemotactic activity, possibly by destabilizing the protein structure. It is likely that the long C-terminal tail of Lungkine could also be involved in stabilizing the structure of the protein.

Lungkine shows the highest sequence identity at the amino acid level, 35%, with human platelet basic protein. ENA-78 (a human CXC chemokine) follows with 31% sequence identity, human IL-8 and mouse MIP-2 with 27%, human growth-regulated proteinα with 26%, and rat cytokine-induced neutrophil chemoattractant-2β (CINC-2β) with 22%.

Chromosomal localization

The initial experiment resulted in specific labeling of the middle portion of a medium-sized chromosome, which was believed to be chromosome 5 on the basis of 4′,6-diamidino-2-phenylindole staining. A second experiment was conducted in which a probe specific for the telomeric region of chromosome 5 was cohybridized with a Lungkine cDNA probe. This experiment resulted in the specific labeling of the telomere and the mid portion of chromosome 5. Measurements of 10 specifically hybridized chromosomes 5 demonstrated that the *Lungkine* gene is located at a position that is 50% of the distance from the heterochromatic-euchromatic boundary to the telomere of chromosome 5. A total of 80 metaphase cells were analyzed, with 67 exhibiting specific labeling (data not shown). Therefore, we conclude that the *Lungkine* gene is located in mouse chromosome 5.

Most CXC chemokine genes are located in human chromosome 4 (4q12–21) and mouse chromosome 5. The clustering of this gene subfamily strongly suggests that all have diverged from a common ancestral gene.

Lungkine is a chemokine specifically expressed in the lung

The results obtained from Southern blot analyses of the cDNA libraries suggested that Lungkine had a very restricted pattern of expression (data not shown). To confirm this, a mouse tissue

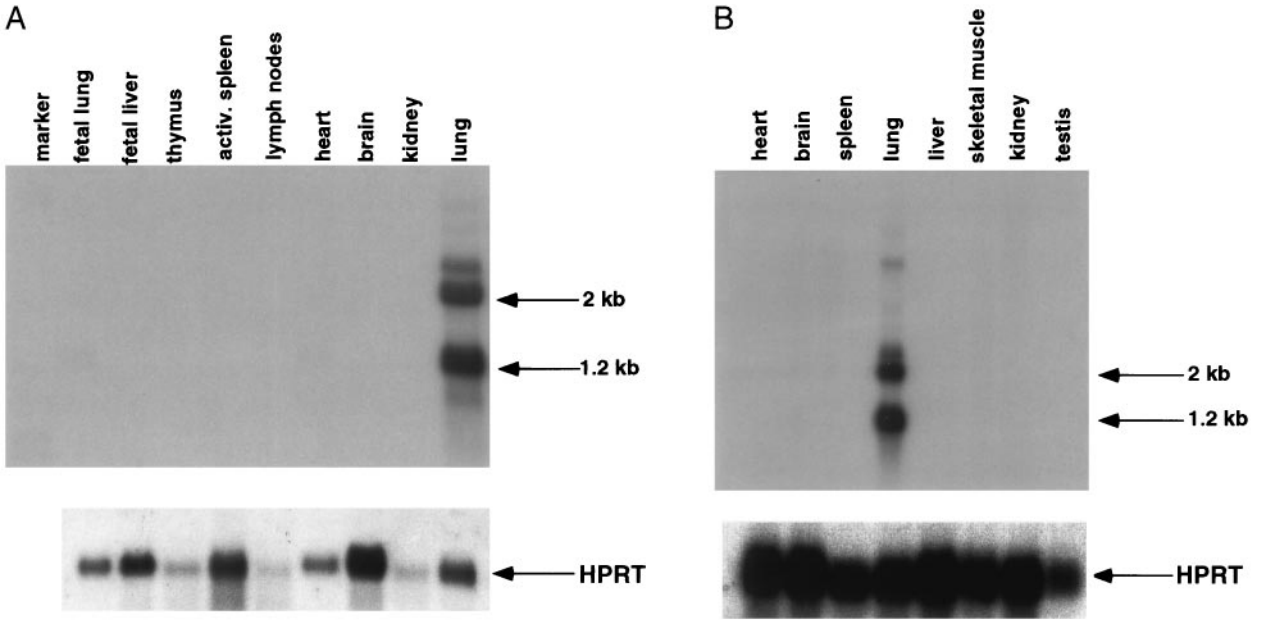

FIGURE 2. Tissue distribution of Lungkine mRNA. *A*, A total of 15 μg of total RNA was loaded on each lane. The Northern blot was hybridized to the Lungkine cDNA probe as outlined in *Materials and Methods*. The same membrane was hybridized to HPRT to normalize RNA loading (*bottom panel*). *B*, The mouse tissue blot (Clontech) was hybridized as described in *A*.

Northern blot and a cell line Northern blot were hybridized with a Lungkine cDNA probe. All of the mouse cell lines tested, including 3D.1 (thymus epithelium), MC9 (mast cell), A3.2 (NK T cell hybridoma), HT-2 (T cell clone), L cells (fibroblast), a prothymocyte T cell hybridoma, a prethymocyte T cell hybridoma, A20-2J (B cell lymphoma), Lewis lung carcinoma cells, as well as the BW5147 T cell thymoma, gave no signal (data not shown). Conversely, the tissue blot showed an intense signal on lung RNA samples (Fig. 2*A*), a weak signal on fetal lung tissue, and a faint signal in the heart. However, other cDNA libraries derived from the total heart or aorta showed no signal for Lungkine (data not shown). The thymus and Con A-stimulated spleen, lymph nodes, brain, kidney, and fetal liver were negative for Lungkine expression. These results agree with the signals detected using a murine tissue Northern blot (Clontech) (Fig. 2*B*). Until now, all other chemokines reported expressed in the lung were also expressed elsewhere. For example, IFN-inducible T cell α chemoattractant (I-TAC) is expressed not only in the lung but also in the pancreas, thymus, spleen, and brain (21). Fractalkine is expressed in the lung, kidney, skeletal muscle, heart, brain, and testis (22). Monocyte chemoattractant protein-5 (MCP-5) is expressed in the lymph nodes, thymus, and lung (23). MCP-4 is constitutively expressed in the small intestine, colon, and lung (24). Macrophage-derived chemokine (MDC) is detected in the thymus, lung, and spleen (25). Other examples include MCP-2 (26), pulmonary and activation-regulated chemokine (PARC) (27), MIP-3α (7, 10, 11), etc. We conclude that the highly specific pattern of expression that Lungkine exhibits is unique.

Lungkine mRNA is up-regulated under inflammatory conditions

Based on the expression data obtained from mouse cDNA libraries and tissue blot analyses, we sought to examine Lungkine mRNA regulation in the inflamed lungs of various animal disease models: an asthma OVA model, an *N. brasiliensis* infection model, and an *Aspergillus* infection model. Lung and mediastinal lymph nodes were obtained from OVA-sensitized BALB/c mice (see *Materials and Methods*) at 3, 6, and 24 h after Ag challenge. Total RNA was prepared, and Northern blot analyses were performed. Fig. 3*A* shows that mediastinal lymph nodes are not responsible for Lungkine mRNA expression. Nevertheless, there was approximately a 2-fold increase in Lungkine mRNA levels in the OVA lung samples 3 h after Ag challenge. The same result was observed with the LPS-treated lung samples at 3 h postinjection. Lungkine mRNA levels were still up-regulated 24 h after the OVA challenge.

Lungs were obtained from *N. brasiliensis*-infected mice at 8 and 10 days postinfection. Lungs from *Aspergillus*-infected mice were also tested 6 h postinfection. Northern blot analysis using total RNA from these samples indicated a 2-fold increase of Lungkine mRNA levels in the *Aspergillus* lungs and in the *Nippostrongylus*-infected lungs at 6 h and at 8–10 days postinfection, respectively (Fig. 3*B*). We observed that OVA-challenged mice as well as *Aspergillus*-infected, LPS-treated, and *N. brasiliensis*-infected mice showed a small but significant increase (2-fold) in Lungkine mRNA expression, suggesting a role for Lungkine in neutrophil trafficking during normal and inflammatory conditions.

Lungkine mRNA is expressed by bronchoepithelial cells

We sought to identify the cellular source of Lungkine. For this purpose, we prepared sections from normal lungs and performed immunohistochemical analyses. The latter detected the presence of Lungkine protein in bronchoepithelial cells (Fig. 4*C*). The negative controls used in this experiment included sections treated with a blocking mix (peptide-Ab) (Fig. 4*B*) or without the primary Ab (Fig. 4*A*).

We have mentioned previously that low levels of Lungkine mRNA were detected in fetal mouse lung tissue by Northern blot analysis. To confirm these results, ISH studies were performed using sections of whole fetal mice from day 16 of gestation. Fig. 5*A* shows a fetal (day 16) lung tissue section hybridized with a Lungkine antisense probe. The bright punctate signal of the silver grains is very clear in Fig. 5*A*; however, this signal was absent when the control sense probe was used (Fig. 5*B*).

In addition, ISHs were conducted to confirm the results from the immunohistochemical and Northern blot analyses. Both normal

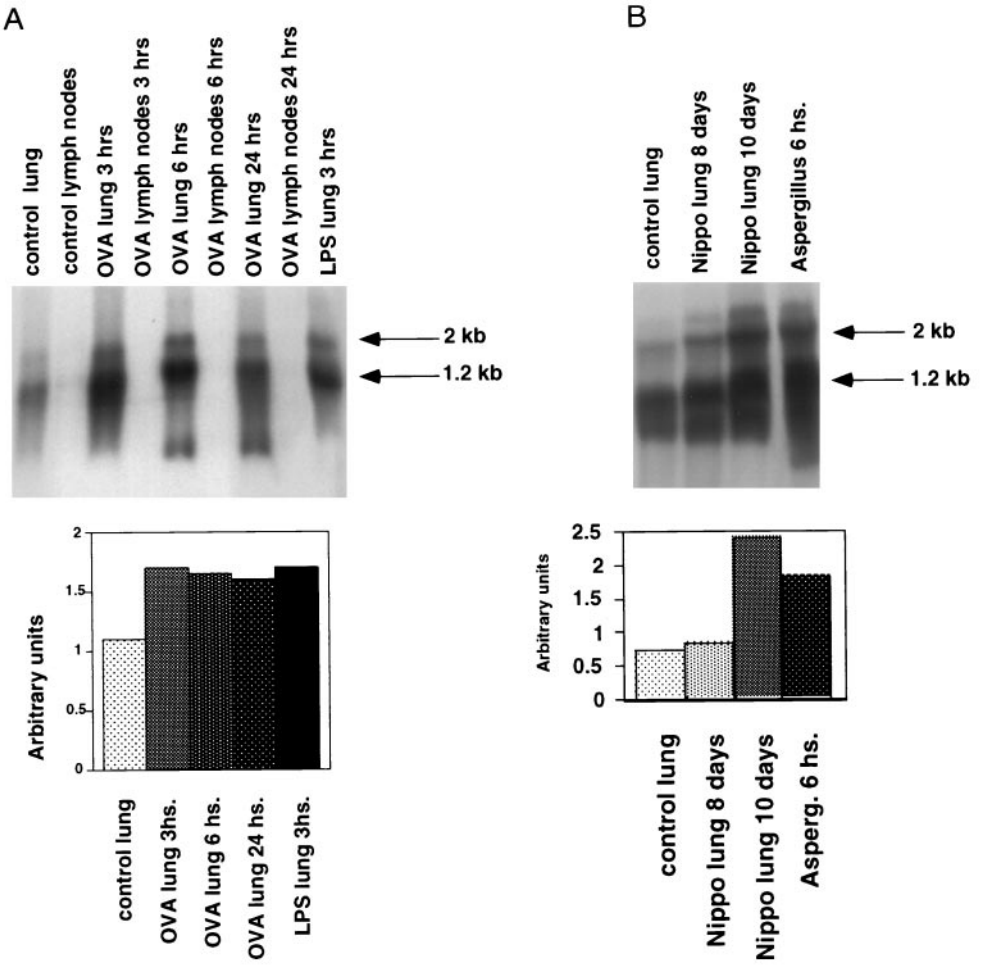

FIGURE 3. Lungkine mRNA expression in murine disease models. *A*, A total of 15 μg of total RNA from mediastinal lymph nodes and lungs of control and OVA-sensitized/challenged BALB/c mice was loaded on the gel, as well as an RNA sample from lungs of LPS-treated mice. The membrane was hybridized to the Lungkine cDNA probe and to an HPRT probe to normalize RNA loading. The quantification of each sample after HPRT normalization is shown in the *bottom panel*. *B*, Total RNA from lungs of *N. brasiliensis*-infected mice, *Aspergillus*-treated mice, and normal mice was loaded on a gel, transferred, and hybridized as outlined in *A*. The quantification is shown in the *bottom panel* after HPRT normalization.

(Fig. 5*C*) and OVA-challenged lung (24 h) sections from BALB/c adult mice (Fig. 5*D*) showed a positive signal in the epithelial cells surrounding the bronchi when hybridized with the Lungkine antisense probe. In agreement with our Northern blot results, a stronger signal was detected in the OVA-treated lungs compared with the unstimulated lung sections.

Lungkine is secreted into the bronchoalveolar space

The content of Lungkine protein present in the BAL of normal BALB/c or OVA-sensitized and challenged mice was compared by Western blot analysis (Fig. 6). Lungkine protein was present in the BALs of both unstimulated and challenged mice. After Ab removal,

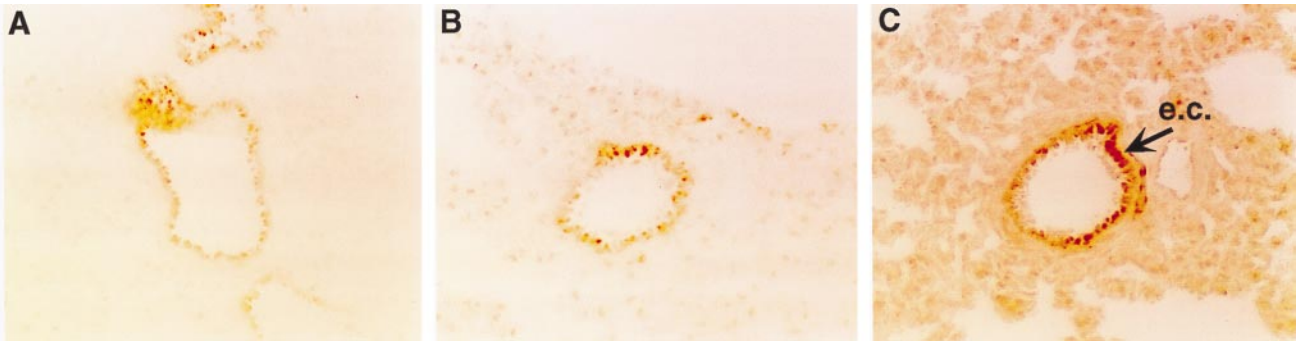

FIGURE 4. Expression of Lungkine protein in vivo. *A–C*, Expression of mouse Lungkine protein in bronchoepithelial cells. Intracellular staining for Lungkine expression with a specific polyclonal Ab was conducted as described in *Materials and Methods*. *A*, Staining with control rabbit antiserum. *B*, Staining with a blocking mix of peptide and anti-Lungkine polyclonal Ab. *C*, Staining with anti-Lungkine polyclonal Ab showing expression by epithelial cells (arrow).

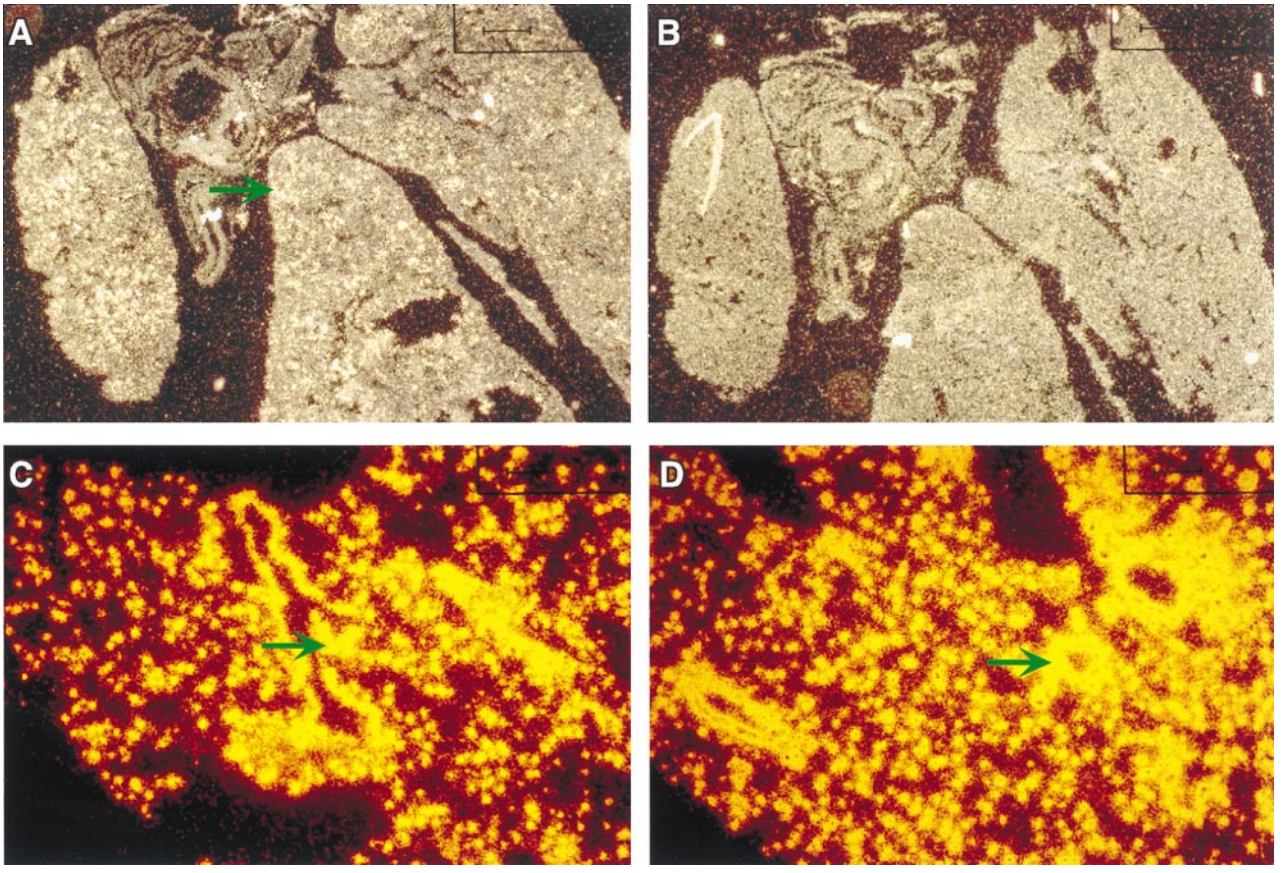

FIGURE 5. Expression of Lungkine mRNA in vivo. *A–D*, Expression of Lungkine mRNA by ISH. ISH was performed as outlined in *Materials and Methods*. *A*, An antisense probe showing expression in a day-16 fetal lung. *B*, Negative control sense probe. *C*, An adult normal lung showing expression on bronchial epithelium (arrow). *D*, An adult OVA-sensitized lung 24 h after Ag challenge (arrow).

the same blots were incubated with rabbit preimmune serum or with an anti-peptide Ab against another chemokine, thymus-expressed chemokine (TECK) (as a control) (data not shown). No signal was detected, confirming the specificity of the anti-peptide Ab against Lungkine.

However, the protein could not be detected in total extracts of unstimulated or LPS-treated lungs. These results indicate that Lungkine is produced by bronchoepithelial cells and is released into the airways. Its absence in total lung extracts may be due to the high number of fibroblasts and endothelial cells vs the lower number of epithelial cells in the lung, which may dilute Lungkine protein in the tissue, preventing its detection.

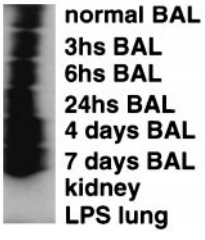

FIGURE 6. Lungkine is present in the BAL. BALs were performed as described in *Materials and Methods* using normal BALB/c mice and OVA-sensitized mice at different timepoints after Ag challenge. BALs from seven BALB/c mice per timepoint were pooled. A total of 10 μg of protein was loaded in each lane. The Western blot was incubated with anti-Lungkine polyclonal Ab. No signal was detected when an anti-mouse thymus-expressed chemokine (TECK) polyclonal Ab or control rabbit serum was used (data not shown).

These results, along with the immunohistochemistry and ISH analyses, confirm that Lungkine is produced by bronchoepithelial cells and indicate that it is released into the airways.

Lungkine is chemotactic for granulocytes

In vivo migration assay. Because Lungkine is an ELR-CXC chemokine, it was likely to be a neutrophil chemoattractant. To test this hypothesis, C3H/HeJ mice (endotoxin-insensitive) were injected i.p. with either 10 μg of Lungkine, with another CXC chemokine, KC (as a positive control), or with PBS. The i.p. injection of Lungkine or KC induced an increase in the number of Gr-1$^+$ cells in the peritoneum (15–20%, respectively) compared with mice injected with PBS (1%) at 3 h postinjection (Fig. 7*A*). By 24 h, the percentage of Gr-1$^+$ cells had decreased significantly (2–4%). However, there was no increase in the percentage of F4/80$^+$ cells in the animals treated with either Lungkine or KC. The percentage of T cells in the peritoneal cavity did not vary significantly between KC, Lungkine, and PBS controls. Similar fold increments were obtained when using BALB/c mice (data not shown).

The in vivo migration assay demonstrated that Lungkine, as a typical ELR-CXC chemokine, induces the migration of neutrophils.

In vitro chemotaxis assay. To confirm the results obtained with the in vivo migration assay, we tested Lungkine in a transwell chemotaxis assay using a cell population enriched in neutrophils (see *Materials and Methods*). Lungkine did induce the migration of neutrophils (Fig. 7*B*). At a concentration of 10^{-5} M, Lungkine induced the migration of 65% of Gr-1$^+$ cells; KC induced the migration of 55%, of these cells. However, 10^{-6} M and 10^{-7} M

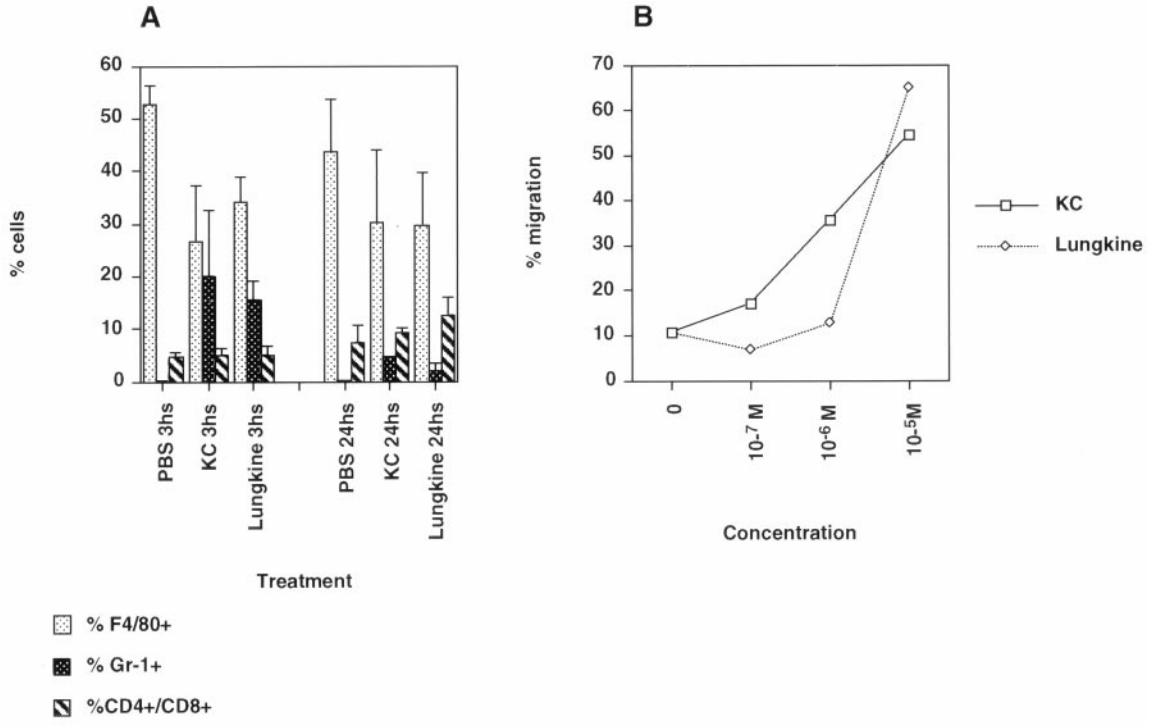

FIGURE 7. In vivo and in vitro migration in response to Lungkine. *A*, In vivo chemotaxis assay. A total of 10 μg of protein (KC and Lungkine) was injected per C3H/HeJ mouse or a similar volume (100 μl) of PBS in the control animals. At 3 and 24 h postinjection, peritoneal lavages were performed. Cells were counted, stained, and analyzed by FACS. The Abs used for staining were Gr-1-FITC, F4/80-PE, and $CD4^+$ and $CD8^+$ tricolor. One representative experiment of three independent assays is shown. *B*, In vitro chemotaxis assay. The transwell chemotaxis assays were performed as described in *Materials and Methods*. One representative experiment of three independent assays is shown.

concentrations of Lungkine did not induce significant cell migration, whereas corresponding concentrations of KC induced only a slightly better response.

Discussion

In this study, we report the discovery of a novel lung-specific chemokine that we have designated Lungkine. The highly specific expression pattern of Lungkine was reflected by the fact that at the time it was discovered, no ESTs encoding this chemokine were present in dbEST. Although other recently reported chemokines such as MIP-3α (7) or pulmonary and activation-regulated chemokine (PARC)/DC-Ck-1 (27) are expressed in the lung as well, they are also expressed elsewhere. Lungkine is the only chemokine whose expression appears restricted to the lung.

We discovered Lungkine while screening a mouse $RAG^{-/-}$ lung cDNA library during a search for the mouse counterpart of MIP-3α. Although Lungkine shares sequence similarity at the amino acid level with some human CXC chemokines (platelet basic protein, ENA-78, IL-8, and others), the degree of sequence similarity is not high enough to unequivocally assign a known human counterpart to Lungkine. Furthermore, none of these chemokines exhibit the characteristic long C-terminal tail of Lungkine. Alternatively, several efforts to identify a human counterpart were attempted, so far yielding negative results. In addition to the screenings of human genomic libraries and of cDNA libraries, Northern blot analyses of human lung cell lines yielded no solid evidence of a human counterpart of Lungkine (data not shown). Finally, no human ESTs for a putative human Lungkine homologue exist in the GenBank dbEST database, despite the fact that the number of human ESTs it contains currently exceeds the number of mouse ESTs by >3-fold. These results, along with the low sequence identity with other known human ELR-CXC chemokines, strongly suggest that human Lungkine, if it exists, will exhibit a highly specific expression pattern. We are currently testing the hypothesis that the expression of human Lungkine may be restricted to certain human inflammatory lung conditions. At present, we conclude that Lungkine represents a novel lung-specific chemokine for which a human homologue has not yet been identified.

Lungkine was found to be expressed by lung epithelial cells (Fig. 5, *C* and *D*). The fact that Lungkine is produced by this cell type and is up-regulated during inflammation suggests a role for this chemokine in leukocyte recruitment to the airways upon Ag challenge.

Similar to other ELR-CXC chemokines, Lungkine induces the in vivo and in vitro migration of neutrophils (Fig. 7, *A* and *B*). Neither T cells nor macrophages migrate to the peritoneal cavity in response to Lungkine between 3 and 24 h after i.p. injection. However, we cannot eliminate the possibility that Lungkine could cause neutrophil activation. Lungkine, as shown for IL-8 (12), could activate neutrophils and generate a T cell migration (72 h after protein injection) by inducing the release of T cell chemoattractants from neutrophils, indirectly affecting another important cell type in asthma.

We hypothesize that Lungkine is involved (along with other chemokines and adhesion molecules) in the homing of neutrophils to protect the airways against exogenous Ags or pathogens. In cases in which certain Ags or pathogens were present in the airways, Lungkine levels would rise, increasing the number of neutrophils recruited to the airways.

ELR-CXC chemokines are known to bind to CXC chemokine receptor 2 (CXCR2) (with the exception of IL-8, which also binds to CXCR1), which is essentially expressed by neutrophils and, to

a lesser extent, by T lymphocytes (28, 29). We have tested Lungkine binding to mouse CXCR2 but have been unable to demonstrate the binding of Lungkine to CXCR2 using either Ca^{2+} flux or binding assays (data not shown).

CXC chemokines have been described as key molecules in the regulation of angiogenesis (30). Whereas ELR-CXC chemokines promote angiogenesis, non-ELR-CXC chemokines suppress it. Lungkine could be involved in the angiogenic processes occurring during embryonic development; in addition its mRNA up-regulation in the adult stage may be necessary to support the growth and development of the lung. Experiments currently underway will aim to test this hypothesis.

Most of the literature has focused on the CC chemokines responsible for the eosinophilic and T lymphocyte infiltration observed during allergic airway inflammation. In contrast, not much information is available on the role of CXC chemokines and neutrophils in allergy and asthma. For example, in sudden-onset fatal asthma cases (also called "sudden asphyxic asthma"), patients have shown a larger number of neutrophils than eosinophils in the airway submucosa (31, 32). These data not only confer relevance to the neutrophilic infiltrate in asthma, but also suggest an important role for neutrophil chemoattractants in this disease.

These data, along with the suggested role of eosinophils and T cells as players in asthma (33–35), broaden our understanding of how chemokines orchestrate allergic airway inflammation. Given that Lungkine represents a new ELR-CXC chemokine that is specifically expressed by lung bronchoepithelial cells, it will be interesting to explore its role both in normal lung homeostasis and in pathological conditions.

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