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CXCL14 Displays Antimicrobial Activity against Respiratory Tract Bacteria and Contributes to Clearance of *Streptococcus pneumoniae* Pulmonary Infection

Chen Dai,* Paola Basilico,* Tiziana Patrizia Cremona,* Paul Collins,† Bernhard Moser,† Charaf Benarafa,*1 and Marlene Wolf*1

CXCL14 is a chemokine with an atypical, yet highly conserved, primary structure characterized by a short N terminus and high sequence identity between human and mouse. Although it induces chemotaxis of mononuclear cells at high concentrations, its physiological role in leukocyte trafficking remains elusive. In contrast, several studies have demonstrated that CXCL14 is a broad-spectrum antimicrobial peptide that is expressed abundantly and constitutively in epithelial tissues. In this study, we further explored the antimicrobial properties of CXCL14 against respiratory pathogens in vitro and in vivo. We found that CXCL14 potently killed *Pseudomonas aeruginosa*, *Streptococcus mitis*, and *Streptococcus pneumoniae* in a dose-dependent manner in part through membrane depolarization and rupture. By performing structure-activity studies, we found that the activity against Gram-negative bacteria was largely associated with the N-terminal peptide CXCL141–13. Interestingly, the central part of the molecule representing the β-sheet also maintained ∼62% killing activity and was sufficient to induce chemotaxis of THP-1 cells. The C-terminal α-helix of CXCL14 had neither antimicrobial nor chemotactic effect. To investigate a physiological function for CXCL14 in innate immunity in vivo, we infected CXCL14-deficient mice with lung pathogens and we found that CXCL14 contributed to enhanced clearance of *Streptococcus pneumoniae*, but not *Pseudomonas aeruginosa*. Our comprehensive studies reflect the complex bactericidal mechanisms of CXCL14, and we propose that different structural features are relevant for the killing of Gram-negative and Gram-positive bacteria. Taken together, our studies show that evolutionary-conserved features of CXCL14 are important for constitutive antimicrobial defenses against pneumonia. *The Journal of Immunology*, 2015, 194: 5980–5989.

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pathic stretches. An initial screening for antimicrobial activity of chemokines revealed that large patches of positive electrostatic charges on the surface is an important characteristic to distinguish antimicrobial from nonantimicrobial chemokines (9), whereas cationic amino acids, which are often clustered in the C-terminal α-helix, are thought to interact with the negatively charged bacterial membranes (7, 8, 24). However, as described for CXCL6 (25) and for thrombomodulin-1, a variant of CXCL7 lacking C-terminal alanine and aspartate (26), there are exceptions where key structural features for antimicrobial activity of chemokines lie in the N-terminal part. It seems that disulfide bonds are not essential for antimicrobial activity of chemokines (7, 27, 28). Thus, it appears that each chemokine executes its antimicrobial activity through variable and individual structural features. Knowledge of the molecular mechanisms of endogenous antimicrobial molecules is important for understanding host self-defense processes and a prerequisite for developing new classes of antibiotics for the treatment of infections.

In this study, we have identified distinct structural elements that are responsible for the antimicrobial and chemotactic activities of CXCL14. We found evidence that CXCL14 induced membrane depolarization and leakage of intracellular contents, and that it bound to bacterial DNA. Furthermore, its in vitro and in vivo activity against lung opportunistic and pathogenic bacteria revealed a role for CXCL14 in innate protection against pulmonary infections.

Materials and Methods

Peptides and microorganisms

Synthetically produced CXCL14 and CXCL8 (29) were used in all experiments unless specifically referred to as rCXCL14, which was from R&D Systems. Transfected CXCL14 were custom synthesized by Thermo Fisher Scientific (Ulm, Germany) or Shanghai Biochemical (Shanghai, China) and designated as follows: CXCL14_1–8 (SKCKCSRK); CXCL14_9–16 (SKCKCSRKRG); CXCL14_17–25 (SKCKCSRKGGP); CXCL14_26–33 (SKCKCSRKGGPKR); CXCL14_34–41 (YSDVKKLEKPKYPICEEKMMVIITKSVSRYGQEHLPK); CXCL14_42–49 (LQSTKRFKIWYNAWNEKR); CXCL14_50–57 (LQSTKRFKIWYNAWNEKRKR); CXCL14_58–65 (LQSTKRFKIWYNAWNEKRYYE). The accurate concentrations of all the peptides were determined by amino acid analysis (Analytical Research and Services, University of Bern, Bern, Switzerland), Human β-defensin (hBD)-2 was from Bachem (Bubendorf, Switzerland), and hBD-3 was from Peptide Institute (Minoh-shi Osaka, Japan). Pseudomonas aeruginosa HER-1018 (PAO1) (ATCC BAA-47) was from LGC Standards. Streptococcus pneumoniae (R&D Systems) according to manufacturer’s instructions. CXCL14 ELISA and LPS binding assay

CXCL14 concentrations in bronchoalveolar lavage (BAL) and in lung homogenates were quantified using a commercially available ELISA kit (R&D Systems) according to manufacturer’s instructions.

LPS binding was performed according to Scott et al. (33) with slight modifications. Ninety-six-well plates were coated with 100 μL LPS from E. coli (Sigma-Aldrich) at 1 μg/ml in RPMI 1640 and incubated at 37°C for at least 5 h. The wells were washed three times with PBS to remove unbound LPS, and the plate was left to air dry overnight at room temperature. The plate was then blocked with 500 μL PBS, 1% BSA for at least 1 h at room temperature, and washed three times with PBS, 0.05% (v/v) Tween 20. A total of 100 μL CXCL14 was added to the wells and incubated for 2 h at room temperature. The washing step was repeated and 100 μL biotinylated mouse anti-human CXCL14 was added to the plate and left 2 h at room temperature. Bound CXCL14 was quantified with streptavidin-coupled HRP as in ELISA measurements.

Chemokine binding to E. coli plasmid DNA

Indicated amounts of CXCL14 or CXCL8 in 20 μl binding buffer (10 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, 20 mM KCl, 50 μg/ml BSA, 5% glycerol, pH 8.0) were incubated with 400 ng plasmid DNA from E. coli in Eppendorf vials for 1 h at room temperature. The reaction was stopped by adding 4 μl loading buffer, and the samples were separated by electrophoresis on 1% agarose gels.

Reduction, linearization, and alklylation of CXCL14

Disulfide bonds of CXCL14 were reduced by incubating 120 μg CXCL14 for 1 h at 50°C in 100 μl 200 mM Tris-HCl, 100 mM DTT, pH 8.0. Bromphenol blue was included as reduction indicator. Then 100 mM iodoacetamide was added and the sample was further incubated at room temperature for 30 min. During this time, the samples were mixed with 10 μl 3 M sodium acetate (pH 5.5) and 10 μl 3 M sodium acetate (pH 5.5) and 10 μl 3 M sodium acetate (pH 5.5) and 10 μl 3 M sodium acetate (pH 5.5) and 10 μl 3 M sodium acetate (pH 5.5).
temperature for 2 h in the dark. Finally, 4 μl formic acid was added and the reduced peptide purified by reversed phase HPLC using a C18 column. The molecular mass was verified by mass spectrometry.

Transwell chemotaxis assay
Human mononuclear THP-1 cells were cultured for 2 d in the presence of 1 μM PGE2 (Sigma-Aldrich) in RPMI 1640 supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 50 μg/ml penicillin/streptomycin, 10% FCS (all Life Technologies), and 50 μM 2-ME (Sigma-Aldrich). Cells were washed and resuspended at 1×10^5 cells/ml in RPMI 1640 containing 1% human serum albumin, and 20 mM HEPES (chemotaxis buffer). The wells of a 96-well culture plate (Corning Life Sciences) were filled with 235 μl chemotaxis buffer containing the indicated concentration of CXCL14 or synthetic peptide. Chemotaxis buffer alone was used as a control for random cell migration (blank). Transwell filters (5-μm pore size; Corning Life Sciences) were placed in the wells, and 80 μl test cells was added to the upper chamber. The plate was incubated for 3 h at 37˚C, and cells that migrated to the lower chamber were collected and counted using AccuCheck Counting Beads (Life Technologies) by flow cytometry.

CXCL14 cleavage by proteinase 3
Proteinase 3 (Sigma-Aldrich) and CXCL14 were incubated at a molar ratio of 1:12.5 in PBS for the indicated times at 37˚C, and the proteolytic reaction was stopped by boiling the samples for 5 min at 95˚C. Proteolytic degradation of CXCL14 was analyzed on 15% Tris SDS-PAGE and staining with Coomassie blue. Samples for subsequent mass-spectrometry analysis were incubated for 15 min. The reaction was stopped by heating at 95˚C, the fragments separated by reversed phase HPLC on a C18 column, and the resulting peaks were collected, lyophilized, and analyzed by mass spectrometry.

Lung infection model
For in vivo studies, P. aeruginosa was freshly grown on TSB agar plates overnight, suspended in PBS, and thoroughly vortexed. Inoculum concentration was estimated by spectrometry at 600 nm based on established standard curve dilutions. S. pneumoniae inoculum was performed by direct injection of frozen glycerol stock of 2×10^6 CFUs in brain-heart infusion medium. In all cases, the exact inoculum was determined each time by plating and CFU counting. Bacterial inoculation and tissue harvest was performed essentially as described previously (34). CXCL14−/− and WT mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg), then inoculated intranasally by pipetting 10 μl per naris.

Mouse tissue and fluid sampling
Mice were sacrificed by isoflurane overdose at indicated time points postinfection for tissue sampling or collection of nasopharyngeal lavage (NPL) and/or BAL fluids. The spleen and the lungs were aseptically removed. The lung lobes were immediately transferred to RNase-free tubes, snap-frozen, and kept at −80˚C until RNA extraction was performed. The lung right lobes and the spleen were homogenized in 1 ml ice-cold PBS for CFU analysis or in lysis buffer (0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, 1 mM CaCl2, and 1 mM MgCl2, pH 7.4) for ELISA measurements using a tissue homogenizer (IKA-WERKE T8.01). Lung and spleen homogenates were then serially diluted and plated in triplicates on TSB agar plates. The lung left lobes were immediately transferred to RNase-free tubes and used for RNA extraction. The quantity and integrity of isolated RNA was determined by NanoDrop 1000 (Thermo) and agarose gel electrophoresis, respectively. A total of 500 ng RNA was reverse-transcribed using the SuperScript III first-strand synthesis system (Invitrogen) using random hexamer primers. Real-time PCR was performed in triplicate using MESA green qPCR MasterMix (Eurogentec) and a Viia 7 Real-Time PCR System (Applied Biosystems, Foster City, CA). All samples were normalized to S16 ribosomal protein mRNA. mRNA levels were expressed as fold changes normalized to uninfected WT mice. Primers were produced by Eurogentec with the following sequences: CXCL14: forward 5′-GAC-AGA-CGG-CAG-GAG-CAC-3′, reverse 5′-CAA-GCA-GCG-CTC-TCT-C-3′; S16: forward 5′-GAT-ATT-CGG-GTC-GTG-A-3′, reverse 5′-TTG-AGA-TGG-GCT-GTG-GA-3′.

Statistical analysis
All statistical analyses were performed using GraphPad PRISM 5.0 software (San Diego, CA). Results are expressed as the mean ± SEM. Pairwise comparisons were analyzed by unpaired t test or Mann–Whitney U test according to data distribution. Differences between multiple treatments were compared by one-way ANOVA followed by Bonferroni comparison test.

Results
CXCL14 displays bactericidal effects against respiratory tract bacteria
Previously, we reported that CXCL14 kills Gram-positive and Gram-negative skin and gut resident bacteria, including E. coli, coagulase-negative Staphylococcus spp., and Staphylococcus aureus (6). This raised the question whether CXCL14 may generally play a role in host defense in barrier epithelial tissues including the respiratory tract. We analyzed the capability of CXCL14 to kill commensal, and opportunistic bacterial species that colonize the nasopharynx, airways, and lungs. Using the colony-forming assay, we found that CXCL14 displayed potent and dose-dependent antimicrobial activity. A concentration of 1 μM CXCL14 induced >99% killing of S. mitis, S. pneumoniae 19F, and P. aeruginosa PA01 as well as of E. coli (Fig. 1). CXCL14 was also able to inhibit the growth of different S. pneumoniae serotypes, S. oralis, S. pseudopneumoniae, Moraxella catarrhalis, and Haemophilus influenzae, albeit with lower potency than S. mitis, S. pneumoniae, and P. aeruginosa (data not shown). Generally, based on CFU values, the antimicrobial activity was less efficient than Gram-positive pathogenic bacteria as compared with Gram-negative species. In our studies, we used synthetically produced CXCL14, but similar results were obtained with rCXCL14 (data not shown).

In comparison with the well-characterized defensin hBD-3, CXCL14 was less effective against the Gram-positive commensal bacteria S. mitis (Fig. 1A) and the Gram-negative opportunistic pathogen P. aeruginosa (Fig. 1C). Conversely, CXCL14 was more potent on a molar basis against the Gram-positive pathogenic S. pneumoniae (Fig. 1B). CXCL8 was included as a negative control and was indeed inactive against S. mitis, S. pneumoniae, and E. coli. However, CXCL8 was consistently able to inhibit the growth of P. aeruginosa at concentrations of ≥0.5 μM. It has been reported that proteolytic processing of chemokines by bacterial proteinases did not interfere with their antibacterial activity (35), and that peptides derived from CXCL8 acquired bacteria killing properties (24). Consequently, we tested whether the unexpected antimicrobial activity of CXCL8 could be due to the presence of truncated CXCL8 peptides that have acquired antimicrobial activity. However, when CXCL8 was analyzed by SDS-PAGE after incubation with P. aeruginosa, we were unable to detect CXCL8 breakdown products, indicating that the observed killing of P. aeruginosa was due to intact CXCL8 (data not shown).

CXCL14 induces membrane depolarization and permeability changes, and binds to bacterial DNA
The detailed mechanism of bacterial killing by AMPs and, in particular, by chemokine AMPs is not fully elucidated, but it is generally believed that membrane disruption can occur through the interaction of positively charged residues in AMPs with the neg-
cells lose their membrane potential with 1 mM CXCL14, whereas 77% of the bacterial wall of the bacteria (31) (Fig. 2A). Our data show that CXCL14, which reports membrane potential-dependent changes in the cell interactions, a stronger binding affinity of CXCL14 to DNA is necessary to observe the same effect, indicating that CXCL14 has DNA-binding abilities of CXCL14, similarly as described for buforin II (39). As shown in Supplemental Fig. 1, CXCL14 interacted with bacterial genomic DNA and was able to influence membrane depolarization by flow cytometry using DiBAC4 (3), which reports membrane potential-dependent changes in the cell wall of the bacteria (31) (Fig. 2A). Our data show that CXCL14 depolarized E. coli in a dose-dependent manner (Fig. 2B, 2C) and that it was more potent than hBD-2. A total of 77% of the bacterial cells lost their membrane potential with 1 μM CXCL14, whereas the same concentration of hBD-2 induced depolarization of only 12.4% of the cells. The loss of E. coli membrane potential occurred very fast and was already detected 15 min after addition of CXCL14 (data not shown).

Transmission electron microscopy allows the direct examination of cell morphology and integrity after CXCL14 treatment. We have found that the addition of both 1 μM CXCL14 and 1 μM hBD-2 induces significant membrane damage. Bacteria incubated with PBS showed an intact and smooth surface, whereas bacteria treated with AMP revealed disrupted plasma membranes and leakage of intracellular materials (Fig. 3).

Although membrane depolarization accompanied by cell permeabilization is an important mechanism for killing of bacteria, other processes may also contribute to the bactericidal activity of CXCL14 (37, 38). Thus, we next investigated the possibility of effects of CXCL14 on intracellular pathways and determined DNA-binding abilities of CXCL14, similarly as described for buforin II (39). As shown in Supplemental Fig. 1, CXCL14 interacted with bacterial genomic DNA and was able to influence its electrophoretic mobility in an agarose gel. We found that a ratio of CXCL14/DNA (weight/weight) >1 was sufficient to induce DNA mobility shift. In contrast, a CXCL8/DNA ratio ≥4 was necessary to observe the same effect, indicating that CXCL14 has substantial binding affinity for bacterial DNA than CXCL8. Because binding of peptides to DNA is based on electrostatic interactions, a stronger binding affinity of CXCL14 to DNA is expected because of the higher net charge of this molecule at pH 7.4 (13.0) compared with CXCL8 (net charge of 4.0). Collectively, these findings support the view that multiple mechanisms, including membrane permeabilization and DNA binding, contribute simultaneously to bactericidal activity of CXCL14 (3).

**Antimicrobial activity of CXCL14 is located in the N-terminal part of the molecule**

Because certain classical AMPs and chemokines share structural characteristics, such as C-terminal α-helix, disulphide bridges, and a high proportion of cationic amino acids, we asked whether the antimicrobial activity of CXCL14 is confined to one or more of these specific structural elements of the molecule (Fig. 4). First, we addressed whether intact disulphide bonds were critical for the observed AMP activity. CXCL14 was therefore treated with DTT and iodoacetamide to reduce and alkylate the SH-groups. Disulphide bonds seemed not to be essential as reduced CXCL14 displayed only slightly diminished antimicrobial potency compared with native CXCL14 (Fig. 5A), a finding also reported for CCL28, another chemokine with known AMP activity and hBD-3 (7, 27).

The C-terminal α-helix of CXCL14 also includes a high proportion of cationic amino acids indicating that this region of the chemokine could be responsible, at least in part, for the antimicrobial activity by binding to negatively charged phospholipids in bacterial membranes. In addition, we tested two CXCL14 peptides representing different parts of CXCL14, namely, the N-terminal loop peptide CXCL141-13 and peptide CXCL1414-54 representing the β-sheets in CXCL14 (Fig. 5A). The CXCL14 peptides CXCL1455-77 and CXCL1455-73 correspond to the C-terminal α-helix, and none of them exhibited antimicrobial activity at 1 μM, which is different from most of the previously described antimicrobial chemokines (7, 40). CXCL1455-77 and CXCL1455-73 exerted activity at only very high concentrations (≥50 μM), suggesting that the region responsible for the efficient killing of E. coli is not located in the C-terminal α-helix. CXCL1455-73 was included because it lacks the two negatively charged glutamic acid residues, which could be responsible for the reduced interaction with bacteria. The peptide representing the core part of CXCL14,
CXCL1414–54, in contrast, displayed some intermediate antimicrobial activity and was able to inhibit 60% of *E. coli* growth at a concentration of 1 μM. Surprisingly, the short N-terminal fragment CXCL141–13 displayed identical dose-dependent killing of *E. coli* as intact CXCL14 (Fig. 5A). Therefore, in contrast with other chemokines with AMP activity, we conclude that the N-terminal part of CXCL14 plays a dominant role in the observed antimicrobial responses.

Next, we wanted to further refine the structure associated with the antimicrobial activity by shortening of the N-terminal CXCL14 peptides, and therefore synthesized CXCL141–11, CXCL141–10, CXCL141–9, and CXCL141–8 peptides. As shown in Fig. 5B, the antimicrobial activity against *E. coli* diminished when the length of the molecule was gradually shortened. However, removing glycine from CXCL141–9 exposed lysine as the C-terminal amino acid in CXCL141–8, and this form regained antimicrobial activity against *E. coli* with similar potency as CXCL141–11, but it was significantly lower than CXCL141–13. The active peptides CXCL141–8, CXCL141–11, and CXCL141–13 have two cysteines as common features, as well as a cationic amino acid (lysine and arginine, respectively) at the C-terminal end. To determine whether cysteines are critical for activity in small peptides, we synthesized mutated variants with alanine in place of cysteine at positions 3 and 5. Cysteines were not absolutely required because mutated CXCL141–13(AA), where the two cysteines were replaced by alanine, retained part of the activity at 1 and 0.5 μM. However, the activity of the alanine mutated CXCL141–11(AA) was totally abolished, suggesting that cysteines are partly important for the bactericidal activity especially with smaller peptides (Fig. 5C). When tested against respiratory tract bacteria, CXCL141–8 and CXCL141–13 revealed bactericidal activity toward *P. aeruginosa*, but were very weak against Gram-positive *S. mitis* and *S. pneumoniae* (Fig. 5D). All peptides were tested for cytotoxicity by determining lactate dehydrogenase release in the human lung epithelial cell line A549, but none was cytotoxic at concentrations up to 2 μM (data not shown).

**CXCL14-mediated chemotaxis is structurally independent of the AMP activity**

CXCL14 is a highly efficient chemoattractant for human blood monocytes at micromolar concentrations (11, 12). To examine whether the peptides with AMP activity also retained chemotactic activity, we performed in vitro chemotaxis experiments using PGE2-treated THP-1 cells as responder cells. As shown in Fig. 6A, 1–3 μM intact CXCL14 induced robust migration responses. By contrast, none of the N-terminal peptides, including the ones with highest AMP activity (CXCL141–13, CXCL141–11),...
were active in the range of 0.1 to 10 μM (Fig. 6B). Similarly, CXCL1455–77, comprising the C-terminal α-helix of CXCL14, was inactive. Only CXCL1414–54, corresponding to the β-sheet region in intact CXCL14, showed moderate but clearly detectable activity at the highest concentration (10 μM) tested. We conclude that the regions defining the AMP and chemotaxis activity do not overlap, which is in line with the proposed multiple-sites model defining chemokine–chemokine receptor interactions (41).

Proteolytic processing with proteinase 3 generates the bactericidal peptide CXCL141–17

Because we found that different peptides derived from the N-terminal portion of CXCL14 have antimicrobial activity, we

**FIGURE 4.** Structure and primary sequence of human CXCL14. Sequences in red of the ribbon diagram correspond to the N-terminal region of (CXCL141–13), the core structure containing three antiparallel β-strands (CXCL1414–54), and the C-terminal α-helix (CXCL1455–77). The primary amino acid sequence of CXCL14 is shown below with four conserved Cys-residues indicated by triangles (▼).

**FIGURE 5.** Peptides derived from the N-terminal part of CXCL14 have antimicrobial activity. Increasing concentrations of reduced and alkylated CXCL14 and of the variants CXCL141–13, CXCL1414–54, CXCL1455–77, and CXCL1455–73 were incubated with *E. coli* and tested for antimicrobial activity (A). A total of 1 μM of the gradually shortened N-terminal CXCL14 peptides, CXCL141–13, CXCL141–11, CXCL141–10, CXCL141–9, and CXCL141–8, was tested for the capacity to kill *E. coli* (B). The two cysteines in CXCL141–13 and CXCL141–8 were replaced by alanine [CXCL141–13(AA) and CXCL141–8(AA), respectively] and tested against *E. coli* (C). (D) CXCL141–8 (left panel) and CXCL141–13 (right panel) were tested against *E. coli*, *S. mitis*, *S. pneumoniae*, and *P. aeruginosa*. Data are from three to seven independent experiments (mean ± SEM). Differences between groups were compared using nonparametric two-tailed Mann–Whitney *U* test or unpaired *t* test or one-way ANOVA Tukey multiple comparison test. *p < 0.05, **p < 0.01, ***p < 0.001.
wondered whether such functionally active peptides may also occur under physiological conditions by proteolytic processing. CXCL14 is constitutively produced in lung epithelial cells, and we tested whether it could be a substrate for proteases released by activated neutrophils that migrate into the lung upon infection (42). In vitro cleavage experiments showed that proteinase 3, one of the main proteases secreted by activated neutrophils, specifically and efficiently processed CXCL14, and several proteolytic fragments were already detectable after 1 min of protease exposure (Fig. 7A). Mass-spectrometry analysis and Edman degradation of the fragments identified a mass corresponding to CXCL141–17. Of note, the antibacterial activity of synthesized CXCL141–17 was highly effective and comparable with full-length CXCL14 against E. coli and P. aeruginosa but was ineffective against the Gram-positive S. mitis and S. pneumoniae, which is in accordance to the restricted activity of CXCL141–13 and related peptides. Our results indicate that not only full-length CXCL14, but also an N-terminal fragment of CXCL14 generated during proteolytic processing was a potent AMP against Gram-negative bacteria (Fig. 7B).

P. aeruginosa infection increases CXCL14 expression in murine lung

Strong expression of CXCL14 was previously reported in the large bronchial epithelial cells and in mouse alveolar macrophages (20). We investigated the level of CXCL14 in murine lung tissue and how it changed in response to bacterial infection. C57BL/6 WT mice were intranasally inoculated with 2 × 10⁶ CFUs/ml P. aeruginosa or with PBS vehicle, and the mice were sacrificed 30 min and 24 h postinfection. CXCL14 protein levels were determined by ELISA in BAL and lung tissue and compared with uninfected animals. First, we noticed that CXCL14 was prominently expressed in untreated mice and mice treated with PBS, both in BAL and lung tissue homogenate, which fully agrees with previous reports (13, 20), supporting a role for CXCL14 in lung tissue immune surveillance. Second, P. aeruginosa infection moderately increased lung CXCL14 expression 24 h postinfection (Fig. 8A). We therefore also analyzed CXCL14 mRNA levels in lung homogenate and found increased levels 24 h postinfection (Fig. 8B) indicating that CXCL14 production may further increase in response to local infection.

CXCL14-deficient mice have impaired bacterial clearance in the lung

To identify an essential function for endogenous CXCL14 in host defense against lung bacterial infections, we infected CXCL14–/– and WT mice with P. aeruginosa and S. pneumoniae. Bacterial load in lung homogenates and in NPL were determined 4 and 16 h postinfection with P. aeruginosa, but we found no difference in the CFU number between CXCL14–/– and WT mice (Fig. 9A, Supplemental Fig. 2A). In contrast, when mice were infected with S. pneumoniae, a significantly higher bacterial load was detected in the lungs of CXCL14–/– mice compared with WT littermates at 16 h but not 4 h postinfection and when bacterial load was...
higher as $1 \times 10^5$ CFUs (Fig. 9B). Bacterial CFUs in NPL, however, were indistinguishable between WT and CXCL14$^{−/−}$ mice (Supplemental Fig. 2B). There was no evidence of systemic infection, as no viable bacteria were detected in spleen homogenates, neither as a result of $P$. aeruginosa nor $S$. pneumoniae infection (data not shown). Our in vivo data reveal that CXCL14 contributes to the clearance of a relevant pulmonary pathogen, which is representative of Gram-positive bacterial infections, where classical AMPs like the defensins are known to be less effective.

### Discussion

Several chemokines exert additional functions besides their main property as chemotactic cytokines. CXCL14 is one of the more recent chemokines whose chemotactic properties have not been fully investigated, and the slow progress is due, in part, to the fact that its receptor is still elusive. However, recent studies by us (6) and other laboratories (5, 9, 10) have clearly demonstrated that CXCL14 is a potent AMP with broad-spectrum activity. In this study, we found that CXCL14 is a highly active AMP against respiratory tract bacteria and that it contributes to bacterial clearance in the lung. Such a function is plausible considering its homeostatic expression and high abundance in epithelial tissues (20). Our previous findings of the antimicrobial properties of CXCL14 against cutaneous bacteria (6) prompted us to propose a model where CXCL14 is crucial in the early stages of an infection and able to destroy bacteria before the recruitment of inflammatory cells. Of course, it is not expected that this model is limited to the skin and should also apply to other barrier tissues with prominent CXCL14 expression. Our finding that CXCL14 contributes to lung immunity is consistent with the high constitutive expression of CXCL14 in a tissue that is constantly exposed to infectious particles from the environment.

In vitro, CXCL14 killed Gram-positive and Gram-negative species representing commensal, opportunistic, or pathogenic respiratory tract bacteria in a dose-dependent manner with a slightly stronger microbicidal effect against Gram-negative species. In contrast, in vivo, it appeared that the antimicrobial activity of CXCL14 is more relevant for Gram-positive bacteria because CXCL14-deficient mice had impaired $S$. pneumoniae clearance compared with WT animals, whereas no differences in the bacterial load of lungs could be detected after $P$. aeruginosa infection. This may reflect the fact that other AMPs expressed in lungs can target Gram-negative bacteria. The higher potency of CXCL14 against Gram-negative bacteria in vitro suggests that the mechanism and the affinity of the interaction of CXCL14 with Gram-positive and Gram-negative bacteria may be different. This view is supported by our data showing that CXCL14 induced a concentration-dependent membrane depolarization of $E$. coli, as measured by uptake of fluorescent dyes, which was not clearly evident with CXCL14-treated Gram-positive $S$. mitis and $S$. pneumoniae.
Because several reports indicated that LPS, lipid A, or lipoteichoic acid could be a target for AMPs (33, 43–45), we also tested whether CXCL14 could bind to LPS, but we could not detect any interaction.

It is not yet clear which specific structural attributes play a key role in ensuring that a chemokine acts as an AMP. It has been found, though, that the different AMPs possess common characteristics including net positive charges, amphipathic patches, β-sheets, or α-helices that define their tertiary structures (9). CXCL14 comprises several of these elements and by disassembling the molecule into different parts, we were able to map the active site to the N-terminal portion. Importantly, no activity was found for the 23-aa peptide CXCL1455–77, comprising the C-terminal α-helix. This finding was unexpected because positively charged α-helices were considered to be an epitome for a chemokine with AMP function (7, 8, 24, 40). Nevertheless, CXCL6 and thrombocidin-1, a truncated form of CXCL7, are other examples of antimicrobial chemokines where the microbicidal effect is mediated by 50- and 15-aa-long N-terminal peptides, respectively (25, 26, 46). Moreover, similar to other chemokines and defensins, CXCL14 displayed antimicrobial activity after linearization (7, 27, 47). hBD-1, for example, exerted significant antimicrobial activity against Gram-positive anaerobic bacteria only after reduction of the disulfide bonds, and truncated C-terminal peptides of hBD-1 with a length of just 7 aa retained their AMP activity (28). It is possible that the AMP effect of CXCL14 is mediated independently by both its tertiary structure and short linear amino acid sequences.

A specific linear sequence could be responsible for the activity of the short N-terminal peptides and explain why the antimicrobial potency was fully conserved in the 13-aa N-terminal part and only slightly reduced in the smaller peptides with a length of up to 8 aa. The killing activity of the truncated peptides was limited to Gram-negative bacteria. Intact CXCL14, however, is also quite potent in killing Gram-positive species, and this effect was evident in vivo indicating that the tertiary structure of CXCL14 could be responsible for interaction with the cell wall of Gram-positive bacteria. The notion that different mechanisms could be responsible for the killing of Gram-positive and Gram-negative bacteria is consistent with a recent study where N-terminal peptides of thrombocidin-1 were compared with the full-size molecule and found to be less active against Gram-positive S. aureus, but equally efficient with E. coli (26). Accordingly, it is logical to assume that the composition of the linear peptide stretch is important in short peptides where three-dimensional structural elements such as positive patches are less likely to occur.

Almost all AMP studies have been performed in vitro and only few in vivo data are available. It is obviously critical to show that a particular AMP contributes to infection control under in vivo conditions. Such important studies are hampered by a number of concerns, including the tissue distribution and level of expression of CXCL14-deficient mice (10% versus expected 25%) at weaning age is worthy of further investigations to determine whether the cause of death is due to reduced immunity in the perinatal period or to other defects such as altered feeding behavior as reported in adult CXCL14-deficient mice (23).

Because AMPs are substrates for proteolytic processing, their contribution to bacterial killing is expected to be modified by the action of local proteases, most notably those released by neutrophils at the site of microbial infections. We have shown in this study that proteinase 3 is able to cleave CXCL14 and to produce the antimicrobial active form CXCL141–17, suggesting that CXCL14 is broken down, at least transiently, into CXCL141–17, at sites of neutrophil recruitment. Obviously, proteolysis is an important modulatory mechanism for controlling or altering the function of not only AMPs but also chemokines and cytokines at large, which is frequently ignored when data from in vivo infection models are discussed.

In summary, we have demonstrated that CXCL14 is a broad-spectrum AMP against pulmonary bacteria in vitro. The mechanism of its activity is complex but includes membrane depolarization and permeabilization, and suggests that different mechanisms are relevant for the killing of Gram-negative and Gram-positive bacteria. Our structure-function analysis revealed the unexpected finding that the bactericidal activity specific for Gram-negative species is contained primarily in the short N-terminal portion of CXCL14 and that chemotactic activity is associated, at least in part, with the central β-sheet of the molecule. Our in vitro data were validated in a lung infection model showing that CXCL14-deficient mice were more susceptible than WT mice to S. pneumoniae infections. We conclude that the constitutively expressed CXCL14 contributes to the innate arm of the immune surveillance system present in barrier tissues.

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Disclosures
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References


