The CXC Chemokine MIG/CXCL9 Is Important in Innate Immunity against *Streptococcus pyogenes*

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Pharyngitis caused by *Streptococcus pyogenes* is one of the most common bacterial infections in humans and is also a starting point for invasive *S. pyogenes* infection. Here, we describe that tonsil fluid from patients with streptococcal pharyngitis contains high amounts of the interferon (IFN)–dependent CXC chemokine known as monokine induced by IFN-γ (MIG)/CXCL9. Also in vitro, inflamed pharyngeal epithelium produced large amounts of MIG/CXCL9 in the presence of bacteria. The CXC chemokines MIG/CXCL9, IFN-inducible protein–10/CXCL10, and IFN-inducible T cell α-chemoattractant/CXCL11 all showed antibacterial activity against *S. pyogenes*, and inhibition of MIG/CXCL9 expression reduced the antibacterial activity at the surface of inflamed pharyngeal cells. *S. pyogenes* of the clinically important M1 serotype secretes the protein streptococcal inhibitor of complement (SIC), which inhibited the antibacterial activity of the chemokines. As exemplified by *S. pyogenes* pharyngitis, the data identify a novel innate defense mechanism against invasive bacteria on epithelial surfaces.

*Streptococcus pyogenes* preferentially colonizes pharyngeal epithelium. Infections originating from this primary site may cause life-threatening sepsis, necrotizing fascitis, and toxic shock syndrome [1]. Despite the potential virulence of *S. pyogenes*, many individuals are healthy carriers of the bacterium in their upper airways, demonstrating that the bacterium can colonize epithelial surfaces without eliciting an inflammatory response.

Dendritic cells, macrophages, and T cells that reside in subepithelial tissues recognize bacterial antigens, resulting in the production of Th1-polarized proinflammatory cytokines, including interferon (IFN)–γ and tumor necrosis factor (TNF)–α [2, 3]. These cytokines cause an inflamed phenotype of epithelial cells, resulting in the production of host defense molecules, including chemokines [4].

Chemokines are divided into 4 groups—XC, CC, CXC, and CX3C—depending on the presence of conserved NH2-terminal cysteine residues [5]. Monokine induced by IFN-γ (MIG)/CXCL9, IFN-inducible protein (IP)–10/CXCL10, and IFN-inducible T cell α-chemoattractant (I-TAC)/CXCL11 belong to the group of CXC chemokines [6–8]. They all share the ability to signal through CXC chemokine receptor 3 (CXCR3), which is present on T cells and NK cells. Ligand binding to the receptor results in the activation and recruitment of these cells to sites of inflammation [9]. In addition, MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11 possess antibacterial activity in vitro [10, 11].

M protein is a surface molecule of *S. pyogenes* [12]. On the basis of sequence variations in this highly polymorphic protein, strains of *S. pyogenes* can be divided into >100 serological subtypes. Among these, strains of the M1 serotype are the most common, including in severe invasive infections [13]. M1 strains secrete a pro-
tein named “streptococcal inhibitor of complement” (SIC) [14], which exhibits extensive sequence variation [15, 16], interferes with complement activation [14, 17], and blocks the activity of antibacterial proteins and peptides [18, 19]. The interference of SIC with these 2 important branches of innate immunity helps to explain the selective advantage of S. pyogenes strains of the M1 serotype.

The present study shows that MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11 kill S. pyogenes and that MIG/CXCL9 in particular is produced at a bactericidal concentration by inflamed pharyngeal cells both in vivo and in vitro. In response, S. pyogenes secretes SIC, which counteracts the antibacterial effect of the chemokines. The elucidation of these mechanisms provides a better understanding of the molecular basis for streptococcal pharyngitis and identifies a novel principle for the protection against invasive bacterial infections on mucosal surfaces.

MATERIALS AND METHODS

Chemicals and reagents. Recombinant human IFN-γ, TNF-α, growth-related oncogene (GRO)-α/CXCL1, interleukin (IL)-8/CXCL8, MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11 peptides; polyclonal goat antibodies against MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11; and ELISAs for the detection of MIG/CXCL9-derived peptides (N-terminus, RNLVPRTES) were synthesized by Innovagen.

Clinical samples. By use of filter paper, tonsil fluids were collected from the tonsil surface of 8 healthy control subjects (no signs of throat infection and negative culture) and 8 patients with clinical signs of streptococcal pharyngitis (fever, redness of the pharynx, and a positive culture for group A streptococci), after receipt of informed consent. The filter papers were weighed before and after the collection, to estimate the volume of fluid. The procedure was approved by the ethics board of Lund University.

Cell culture. A human pharyngeal epithelial cell line (Detroit 562; American Type Culture Collection) was cultured in minimal essential medium with Earle’s salt (MEM; ICN) supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin (100 U/mL and 0.1 µg/mL, respectively) at 37 °C in an atmosphere containing 5% CO2 with 100% relative humidity. In some experiments, the antibiotics were omitted.

Bacterial strain and purification of SIC protein. The S. pyogenes strain AP1 of serotype M1 was obtained from the World Health Organization Collaborating Center for Reference and Research on Streptococci (Prague, Czech Republic). SIC protein was purified as described elsewhere [14].

Bactericidal assay. AP1 bacteria were grown to mid-log phase in Todd-Hewitt (TH) broth, washed, and diluted in incubation buffer. Fifty microliters of bacteria (2 × 10^6 cfu/mL) was incubated together with chemokines at various concentra-
Figure 1. Presence of chemokines at the tonsillar surface during streptococcal pharyngitis and their production by inflamed epithelial cells in vitro. A, Large amounts of monokine induced by interferon (IFN)-γ (MIG)/CXCL9 in tonsil fluid during streptococcal pharyngitis. Swabs were used to collect fluid from the surface of tonsils, and the chemokine content was determined by ELISA. Samples were taken either from patients with pharyngitis caused by Streptococcus pyogenes (n = 8) or from healthy control subjects (n = 8). B, Parallel between production of MIG/CXCL9, IFN-inducible protein (IP)-10/CXCL10, and IFN-inducible T cell α-chemoattractant (I-TAC)/CXCL11 by inflamed pharyngeal cells in vitro and that in vivo. A human pharyngeal epithelium cell line (Detroit 562) was used to investigate the production of MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11 on stimulation with proinflammatory cytokines. The cell line was grown to confluence and subsequently stimulated with IFN-γ (100 U/mL) and tumor necrosis factor–α (10 ng/mL) for 96 h. The content of MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11 in the cell culture medium was determined by ELISA. Data are mean ± SE values from 3 independent experiments. C–E, Increased production of MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11 by inflamed pharyngeal cells in response to S. pyogenes. Detroit 562 cells were incubated with IFN-γ (100 U/mL) in the absence (white boxes) or presence (black circles) of heat-killed S. pyogenes bacteria of the AP1 strain (1 × 10⁸ cfu/mL). The concentration of MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11 was determined in the cell culture medium by ELISA at the time points indicated. Data are mean ± SE values from 3 separate experiments.
Figure 2. Analysis of the bactericidal activity of chemokines. A–C, Antibacterial activity of recombinant monokine induced by interferon (IFN)-γ (MIG)/CXCL9, IFN-inducible protein (IP)-10/CXCL10, and IFN-inducible T cell α-chemoattractant (I-TAC)/CXCL11 against Streptococcus pyogenes (AP1 strain). Bacteria (50 μL; 2 × 10⁶ cfu/mL) were incubated with the chemokines at the concentrations indicated for 2 h at 37°C. Bacterial killing (in percentage) was determined by comparing the no. of bacterial colonies after exposure to chemokines with the no. obtained after incubation in buffer alone. Data are mean ± SE values from 4–7 separate experiments. D, Bacterial killing in the absence or presence of sodium chloride (150 mmol/L). S. pyogenes (AP1 strain) (50 μL; 2 × 10⁶ cfu/mL) was incubated with MIG/CXCL9, IP-10/CXCL10, or I-TAC/CXCL11 (0.2 μmol/L) as described for panels A–C. Data are mean ± SE values from 4 separate experiments. E, Structure prediction of MIG/CXCL9 showing an NH₂-terminal region containing 3 antiparallel β strands and an extended α-helix in the COOH-terminal part of the molecule. Two peptides were synthesized spanning cationic regions in the NH₂-terminal (red) and COOH-terminal (green) regions. The sequences of the peptides and their isoelectric points (pI) are indicated in the figure. F, Separate incubation of the NH₂- and COOH-terminal peptides with S. pyogenes (AP1 strain) (50 μL; 2 × 10⁶ cfu/mL) for 2 h at 37°C. Bacterial killing was determined by comparing the no. of colony-forming units after incubation in medium alone. Data are mean ± SE values from 3 separate experiments.
the coated surfaces. The association ($k_a$) and dissociation ($k_d$) rate constants were determined simultaneously using the equation for 1:1 Langmuir binding in the BIA Evaluation software (version 3.1; BIAcore). In experiments in which possible interference from SIC of the heparin-binding properties of MIG/CXCL9 was investigated, heparin was bound on the surface of a CM5 sensor chip. First, streptavidin (Pierce) was immobilized via amine coupling. Biotinylated heparin (Sigma) was then injected over the streptavidin surface and immobilized to ~150 resonance units. MIG/CXCL9 was injected over the 2 cells at different concentrations (31–250 nmol/L). For experiments using SIC as an inhibitor of MIG binding, an intermediate but still plateau-forming response concentration of MIG/CXCL9 (63 nmol/L) was chosen. MIG/CXCL9 was preincubated with increasing (10–320 times molar ratios) SIC concentrations.

**Molecular modeling.** A model structure of full-length MIG/CXCL9 (Q07325) was created on the basis of a theoretical model of MIG/CXCL9 [23]. This model contains residues 30–91 and was built using the nuclear magnetic resonance structure of truncated human macrophage inflammatory protein 2α (GRO-β/CXCL2) with Protein Data Bank code 1QNK [24] as template. The model was extended in the N-terminus by residues Thr23 to Gly29, built in an extended conformation. Similarly, the COOH-terminus was extended by residues Gln92 to Thr125, but built in an α-helical conformation. A short energy minimization and molecular dynamics simulation completed the full-length model (MacroModel Module; Schrödinger).

The Robetta full-chain protein structure prediction server was used to predict the structure of the protein SIC [25]. SIC was predicted to contain 2 domains: the first NH2-terminal 205 residues (33–237 of Q54958) in the first domain, and the last 68 COOH-terminal residues in the second domain. No suitable template existed for either of the domains.

**RNA interference.** Inhibition of MIG/CXCL9 production was achieved using siRNA (Stealth siRNA; Invitrogen), in accordance with the manufacturer’s instructions. The kit included RNA duplex oligoribonucleotides against human MIG/CXCL9 (GGU AUU GCC AAU AAU UUG CUC GCC U and AAG GGA GCA AAU UAU UAG GUA UAC C), control RNA (GGU UCC AAU AAU UUA CUC GCC ACU U and AAG UAG CGA GUA AAU UAU UGG AAC C), and siRNA transfection reagent (Lipofectamine).

Briefly, Detroit 562 cells were seeded in 24-well plates without antibiotics. In selected wells, cultures were transfected with oligoribonucleotides directed against MIG/CXCL9 or control siRNA, respectively, when 30%–50% confluent. After 4–6 h, the medium was changed, and the cells were grown until confluence. Thereafter, cells were incubated in the presence or absence of stimuli (IFN-γ at 100 U/mL and TNF-α at 10 ng/mL) for 24 h, followed by incubation with bacteria and viable count assay, as described above.

**Statistical analysis.** Statistical significance was determined on the basis of Student’s $t$ test for paired observations.

**RESULTS**

**Presence of MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11 in tonsil fluid during streptococcal pharyngitis and production of these chemokines by inflamed pharyngeal cells in vitro.**

Fluid was collected from the epithelial surface of tonsils of patients with *S. pyogenes* pharyngitis ($n = 8$). As controls, samples of tonsil fluid from healthy volunteers were collected ($n = 8$). In the patient samples, MIG/CXCL9 was present at the highest levels, whereas IP-10/CXCL10 and I-TAC/CXCL11 were detected at lower concentrations, as determined by ELISA (figure 1A). In the control samples, the chemokines were either undetectable or present at low concentrations.

To investigate whether inflamed pharyngeal epithelial cells produce MIG/CXCL9, IP-10/CXCL10, or I-TAC/CXCL11 in vitro, a cell line (Detroit 562) derived from pharyngeal epithelium was used. Bacterial infection induces a Th1 cytokine profile, in which IFN-γ and TNF-α are key cytokines; therefore, a combination of these, or IFN-γ alone, was used. Analogous to what was found in vivo, MIG/CXCL9 was the predominant chemokine in the supernatants (figure 1B). Western blotting of supernatants showed a single band corresponding to the intact MIG/CXCL9 peptide (data not shown).

IFN-γ-stimulated epithelial cells increased their production of MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11 2–4-fold in the presence of heat-killed *S. pyogenes* (figure 2A–2C). MIG/CXCL9 was the most potent, with an ED$_{50}$ of 0.022 ± 0.005 μmol/L (mean ± SE from 5 independent experiments), whereas the ED$_{50}$ for IP-10/CXCL10 and I-TAC/CXCL11 was 0.17 ± 0.03 μmol/L and 0.17 ± 0.02 μmol/L, respectively (mean ± SE from 3 independent experiments).

Many antibacterial peptides are sensitive to salt, but the activity of the chemokines was not significantly affected by sodium chloride at plasma concentration (150 mmol/L) (figure 2D). Antibacterial peptides are cationic, and the MIG/CXCL9 sequence was analyzed to identify regions with high isoelectric points (pls). Two such regions were identified, one in the NH$_2$-terminal and one in the COOH-terminal part of the molecule (figure 2E). Peptides spanning these regions were synthesized and tested in the bactericidal assay against *S. pyogenes*. The peptide derived from the NH$_2$-terminal sequence had no antibacterial activity, whereas the antibacterial activity of the
Figure 3. Inhibition of the antibacterial, but not the chemotactic, activity of monokine induced by interferon (IFN)-γ (MIG)/CXCL9, IFN-inducible protein (IP)-10/CXCL10, and IFN-inducible T cell α-chemotactic (I-TAC)/CXCL11 by the streptococcal inhibitor of complement (SIC) protein. A, Incubation of Streptococcus pyogenes (strain AP1) (50 μL; 2 × 10⁶ cfu/mL) with MIG/CXCL9 (0.2 μmol/L) in the absence or presence of SIC at the concentrations indicated. Data are mean ± SE values from 4 separate experiments. B, Determination of the antibacterial activity of MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11 (all at 0.2 μmol/L) against S. pyogenes (strain AP1) (50 μL; 2 × 10⁶ cfu/mL) in the absence or presence of SIC (1 μmol/L). Student’s t test for paired observations was used to calculate the P values (∗; ***). C, Injection of MIG/CXCL9 at different concentrations (31–250 nmol/L) over SIC immobilized on a BIAcore CM5 sensor chip. The injection was started at (indicated by the left arrow) and was replaced by running buffer after the binding plateaus (indicated by the right arrow at 100 s), resulting in dissociation of bound peptide. Binding parameters were determined, and the dissociation rate constant was calculated to be 145 nmol/L. Data from 1 representative experiment are shown. D, Investigation of the effect of SIC on the chemotactic activity of the chemokines, by use of a chemotactic assay with cells transfected with CXC chemokine receptor 3 (CXCR3), the MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11 receptor. The chemokines (50 nmol/L) were preincubated with buffer alone or with SIC (1.6 mmol/L) before the chemotactic assay. Cells were allowed to migrate in a modified Boyden chamber with a filter pore size of 5 μm for 5 h. Data are mean ± SE values from 3 separate experiments. E, Electron micrographs showing S. pyogenes exposed to MIG/CXCL9 in the absence (left) or presence (right) of SIC. MIG/CXCL9 caused protrusions and leakage of cellular content from the bacteria (left; arrows), which was not seen with SIC present (right). MIG/CXCL9 was labeled with 5-nm colloidal gold, and the left insert shows MIG/CXCL9 in association with lysed bacteria. The insert on the right electron micrograph shows gold-labeled MIG/CXCL9 associated with M proteins on the surface of intact bacteria. RU, resonance units.
An IC50 was seen in SIC, purified from the activity of chemokines by SIC protein.

**Inhibition of the antibacterial, but not the chemotactic activity of chemokines by SIC protein.** SIC, purified from the AP1 strain of the M1 serotype, neutralized the antibacterial activity of MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11 (figure 3A–3B). The molar ratio of SIC versus MIG/CXCL9 at an IC50 was ~1:50. To determine the binding affinities between SIC and MIG/CXCL9, surface plasmon resonance was used (figure 3C). MIG/CXCL9 bound to SIC with a kₐ of 145 nmol/L. The classic antibacterial peptide LL-37 showed similar affinity, whereas the CXC chemokines GRO-α/CXCL1 and IL-8/CXCL8 did not interact with SIC.

To investigate whether SIC interferes with the chemotactic activity of MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11, a cell line stably transfected with the CXCR3 receptor was used (figure 3D). Each chemokine (50 nmol/L) was preincubated with an excess of SIC (1.6 mmol/L), but the chemotactic activity of the chemokines was not significantly affected.

Electron microscopy was used to study whether SIC influenced MIG/CXCL9-induced killing of S. pyogenes. MIG/CXCL9 was labeled with colloidal gold and added to bacteria in either the absence or presence of SIC. Bacteria incubated with MIG/CXCL9 showed protrusions and leakage of cellular contents as well as an association between gold particles and the bacterial surface. In contrast, preincubation of MIG/CXCL9 with SIC preserved the integrity of the bacterial cell wall (figure 3E).

**Further characterization of the binding between MIG/CXCL9 and SIC.** Most antibacterial peptides and proteins show affinity for heparin, a property they share with many chemokines [26, 27]. To investigate whether this is true for MIG/CXCL9 as well, heparin was immobilized on a sensor chip. MIG/CXCL9 bound heparin with high affinity, and preincubation of MIG/CXCL9 with SIC caused a dose-dependent inhibition of this interaction (figure 4A), showing that heparin and SIC compete for the same binding site in MIG/CXCL9.

To investigate which regions of SIC interact with MIG/CXCL9, SIC-derived peptides with low pIs—SIC I (pI, 3.29) and SIC II (pI, 3.84)—were used in the bactericidal assay, with MIG/CXCL9 present at 0.2 μmol/L (figure 4B). At this bactericidal concentration of MIG/CXCL9 (figure 2A), the mean ± SE IC₅₀ of SIC I and SIC II was 0.64 ± 0.09 and 0.054 ± 0.007 nmol/L, respectively. No additive effects were found when the 2 SIC-derived peptides were combined. This contrasts with the much lower IC₅₀ of the SIC holoprotein (mean ± SE, 0.004 ± 0.002 μmol/L) (figure 4B).

**Antibacterial activity of inflamed pharyngeal epithelium expressing MIG/CXCL9.** Confluent Detroit 562 cells were stimulated with IFN-γ and TNF-α. The medium, tested in the bactericidal assay, showed antibacterial activity against S. pyogenes (figure 5A), and the addition of SIC caused a significant dose-dependent reduction of this activity.

In another set of experiments, confluent cells were first incubated in medium alone (resting) or stimulated with a combination of IFN-γ and TNF-α (inflamed) for 72 h. The cell culture medium was discarded, and S. pyogenes bacteria were

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**Figure 4.** Characterization of the interaction between monokine induced by interferon (IFN)-γ (MIG)/CXCL9 and streptococcal inhibitor of complement (SIC). A, Investigation of the affinity between heparin and MIG/CXCL9. Surface plasmon resonance was used to investigate the affinity (upper panel). Heparin was immobilized, and MIG/CXCL9 was injected over the surface of the chip. The injection was started at t = 0 (indicated by the left arrow) and was replaced by running buffer after the binding plateaus (indicated by the right arrow at 100 s), resulting in dissociation of bound peptide. To investigate whether SIC interferes with the binding of MIG/CXCL9 to heparin, MIG/CXCL9 (63 nmol/L) was preincubated (1 h at room temperature) with equimolar or excess concentrations of SIC (10–320 times SIC/MIG molar ratios, respectively). The SIC/MIG mixtures were injected, and steady state binding response plateaus decreased in amplitude with increasing SIC concentrations (lower panel). B, Anionic peptides in the SIC sequence.

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Figure 5. Demonstration that pharyngeal epithelial cells in an inflammatory state are antibacterial. A, Incubation of cell culture medium from pharyngeal epithelial cells, stimulated with interferon (IFN–γ) (100 U/mL) and tumor necrosis factor (TNF–α) (10 ng/mL) for 96 h, with Streptococcus pyogenes (AP1 strain) (50 μL; 2 × 10^5 cfu/mL) for 2 h at 37°C in the absence or presence of SIC at the concentrations indicated. Bacterial killing was measured using viable counts. Student’s t test for paired observations was used to calculate statistical differences (**P < 0.005). B, Incubation of pharyngeal epithelial cells in medium alone (control) or stimulated with IFN–γ (100 U/mL) and TNF–α (10 ng/mL) for 72 h. Thereafter, the cell culture medium was discarded, and S. pyogenes (AP1 strain) (25 μL; 2 × 10^5 cfu/mL) bacteria were applied on top of the epithelial cell layer and incubated for 2 h at 37°C. This was followed by lysis of the epithelial cells and determination of bacterial killing by viable counts. Student’s t test for paired observations was used to calculate statistical differences (****P < 0.0005). C, Scanning electron microscopy of epithelial cells and streptococci incubated as described for panel B. The top electron micrograph shows intact streptococci on the surface of resting epithelium, whereas bacteria on the surface of inflamed epithelium were partly lysed (figure 5C). D, Subjection of pharyngeal epithelial cells to transfection with nonrelevent control siRNA or siRNA directed against MIG/CXCL9 mRNA. The cells were grown to confluence and either incubated in medium alone or stimulated with IFN–γ and TNF–α for 24 h. The medium was collected, and S. pyogenes bacteria (AP1 strain) (25 μL; 2 × 10^5 cfu/mL) were applied on top of the epithelial cells and incubated for 2 h at 37°C, followed by determination of viable counts. The MIG/CXCL9 content of supernatants and the bacterial killing on the cell surfaces were compared between cells subjected to MIG/CXCL9 knockdown and those transfected with control siRNA. Data are mean ± SE values from 5 independent experiments (****P < 0.0005).

layered on top of the epithelial cells. After 2 h of incubation at 37°C, viable counts were determined (figure 5B), and a strong antibacterial activity was recorded. Scanning electron microscopy showed intact bacteria on the surface of resting epithelium, whereas bacteria on the surface of inflamed epithelium were partly lysed (figure 5C). Down-regulation of MIG/CXCL9 production by siRNA showed a strong inhibitory effect on the killing of S. pyogenes at the surface of stimulated pharyngeal epithelial cells (figure 5D). The levels of IP-10/CXCL10 were not affected by this procedure, indicating specific inhibition of MIG/CXCL9 expression (data not shown). Collectively, these data demonstrate that an inflammatory response by epithelial...
cells resulting in the production of MIG/CXCL9 represents a powerful local host defense mechanism against colonizing S. pyogenes.

**DISCUSSION**

In the present study, CXC chemokines were detected at the surface of tonsils during S. pyogenes pharyngitis, and, in the case of MIG/CXCL9, at concentrations that exceed those required to kill S. pyogenes in vitro. In addition, IFN-γ–stimulated epithelial cells were found to respond to incubation with S. pyogenes by the production of bactericidal quantities of chemokines. These data suggest that such an epithelial response in the pharynx to infecting S. pyogenes represents an important host defense mechanism.

In vitro, recombinant MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11 all showed antibacterial activity. However, MIG/CXCL9 was a 10-fold more potent than IP-10/CXCL10 and I-TAC/CXCL11. The higher production and more potent antibacterial activity of MIG/CXCL9 suggest that MIG/CXCL9 is more important than IP-10/CXCL10 and I-TAC/CXCL11 as an innate antibiotic in the pharynx. This was further underlined by the knockdown of MIG/CXCL9 production by use of an siRNA technique, which markedly reduced epithelial surface–associated killing of S. pyogenes. Taken together, the results demonstrate an important protective role for MIG/CXCL9 on inflamed epithelial surfaces.

Previous work [10] has demonstrated that MIG/CXCL9 and IP-10/CXCL10 are antibacterial against *Escherichia coli* and *Listeria monocytogenes*, and, analogous to human defensins, the antimicrobial activity of the chemokines against these bacteria was sensitive to salt (50 mmol/L sodium chloride). In contrast, the present study shows that the activity of MIG/CXCL9, IP-10/CXCL10, and I-TAC/CXCL11 against S. pyogenes was only slightly affected by the presence of 150 mmol/L sodium chloride. Moreover, nonstimulated oral fluid contains only 5 mmol/L sodium chloride [28], suggesting that chemokines possess broad antibacterial activity in the pharynx.

By use of synthetic peptides, the antibacterial activity of MIG/CXCL9 was mapped to the COOH-terminal region of the peptide. This region is cationic and amphipathic and has a predicted α-helical structure—properties that are typical of peptide sequences exhibiting antibacterial activity. When the IP-10/CXCL10 and I-TAC/CXCL11 sequences were analyzed, similar but less pronounced features were identified, and their COOH-terminal α-helices were found to be shorter [29, 30] than the predicted α-helix in MIG/CXCL9. These differences may explain their lower antibacterial activity against S. pyogenes.

As judged from the analysis of tonsil fluid, the concentration of MIG/CXCL9 is high in the pharynx during S. pyogenes pharyngitis. That MIG/CXCL9 has affinity for heparin suggests that the local concentration may be even higher. Thus, interactions with glucosaminoglycans (GAGs) should retain MIG/CXCL9 at epithelial surfaces and further increase the concentration. It has been suggested that binding of chemokines to GAGs may serve as a chemotactic gradient to recruit leukocytes during inflammation [27]. However, the MIG/CXCL9 concentration in tonsil fluid during S. pyogenes pharyngitis by far exceeds that required to induce chemotactic responses [20], indicating that chemokines bound to GAGs on epithelial surfaces could also provide an antibacterial barrier.

SIC [14] is produced and secreted by S. pyogenes strains of the M1 serotype (genes related to *sic* have been found in M12 and M57 strains). SIC interferes with complement function [14, 17] and classical antibacterial proteins and peptides, such as lysozyme, LL-37, and α-defensin [18, 19]. Moreover, inactivation of the *sic* gene significantly impaired the ability of M1 S. pyogenes to colonize mucosal surfaces in an animal model of infection [31], and SIC was also reported to inhibit bacterial adherence and internalization into human epithelial cells [32]. These properties of SIC—and perhaps in particular the finding here that SIC blocks the antibacterial activity of chemokines produced by inflamed epithelium—could provide a powerful selective advantage and help explain the abundance and virulence of the M1 serotype.

As discussed above, the antibacterial activity of the chemokines is most likely located in their cationic α-helical COOH-terminal regions, which should readily interact with the negatively charged SIC. The observation that synthetic peptides from the most anionic regions of SIC were much less efficient inhibitors than intact SIC probably reflects a more effective steric interference by the larger holoprotein. It is noteworthy that SIC also completely blocks the antibacterial activity when the chemokines are present in molar excess. However, many chemokines form oligomers in solution, and it has been suggested that oligomerization promotes their binding to GAGs [29]. In gel filtration experiments (data not shown), we found that MIG/CXCL9 forms oligomers. Interference with such MIG/CXCL9 oligomers in solution could explain why the antibacterial activity is blocked by SIC at a molar excess of MIG/CXCL9. It also raises the interesting possibility that SIC binds to and inactivates antibacterial MIG/CXCL9 multimers associated with GAGs at the epithelial surface.

In contrast to the inactivation of the antibacterial function of chemokines, SIC did not influence their chemotactic activity. Mutational analysis of the interaction between IP-10/CXCL10 and its receptor, CXCR3, did not identify a distinct region in IP-10/CXCL10 responsible for receptor binding and activation, but residues in the NH2-terminal part appeared to be the most important [33, 34]. This is in agreement with the binding of SIC to the antibacterial COOH-terminal region of the chemokines.

S. pyogenes can invade epithelial cells, which may be a way
for the bacterium to escape the effect of antibacterial chemokines [35]. To our knowledge, it has not been investigated whether antibacterial peptides accumulate in endosomes of inflamed epithelial cells.

The finding that inflamed epithelium produces antibacterial chemokines represents a novel innate immune mechanism, one that has been demonstrated here to play a role in the pathogenesis of S. pyogenes pharyngitis. It is likely that this mechanism is not restricted to S. pyogenes infections but, rather, is part of a general defense strategy against colonizing pathogenic bacteria.

Acknowledgments

We thank Ms. Ingrid Gustafsson, Ms. Pia Andersson, and Ms. Maria Baumgarten, for skilful technical assistance, and Dr. Bjorn Walse, for modeling predictive molecular structures.

References