Protein FOG is a moderate inducer of MIG/CXCL9, and group G streptococci are more tolerant than group A streptococci to this chemokine's antibacterial effect

Helena M. Linge,^{1,3} Inka Sastalla,² D. Patric Nitsche-Schmitz,² Arne Egesten³ and Inga-Maria Frick¹

Correspondence

Helena M. Linge

Helena_M.Johansson@med.lu.se

¹Department of Clinical Sciences Lund, Division of Infection Medicine, Lund University, BMC, B14, SE-221 84 Lund, Sweden

²Helmholtz Zentrum für Infektionsforschung, Abteilung Mikrobielle Pathogenität, Inhoffenstrasse 7, D-381 24 Braunschweig, Germany

³Department of Clinical Sciences Lund, Division of Respiratory Medicine and Allergology, Lund University, BMC, B14, SE-221 84 Lund, Sweden

Streptococcus dysgalactiae subsp. *equisimilis* (group G streptococci; GGS) cause disease in humans but are often regarded as commensals in comparison with *Streptococcus pyogenes* (group A streptococci; GAS). The current study investigated the degree and kinetics of the innate immune response elicited by the two species. This was assessed as expression of the chemokine MIG/CXCL9 and bacterial susceptibility to its bactericidal effect. No significant difference in MIG/CXCL9 expression from THP-1 or Detroit 562 cells was observed when comparing whole GGS or GAS as stimuli. The study demonstrates that protein FOG was released from the bacterial surface directly and by neutrophil elastase. Expression of MIG/CXCL9 following stimulation with soluble M proteins of the two species (the recently described protein FOG of GGS and protein M1 of GAS) was reduced for protein FOG in both the monocytic and the epithelial cell line. When the antibacterial effects of MIG/CXCL9 were examined in conditions of increased ionic strength, MIG/CXCL9 killed GAS more efficiently than GGS. Also in the absence of MIG/CXCL9, GGS were more tolerant to increased salt concentrations than GAS. In summary, both GGS and GAS evoke MIG/CXCL9 expression but they differ in susceptibility to its antibacterial effects. This may in part explain the success of GGS as a commensal and its potential as a pathogen.

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INTRODUCTION

The epithelial linings of the human dermis and upper airways are the outermost line of human defence against a plethora of pathogens and a common site of isolation of group G streptococci (GGS; *Streptococcus dysgalactiae* subsp. *equisimilis*). These Gram-positive cocci have traditionally been considered as commensals colonizing epithelial surfaces, but their potential as emerging human pathogens has recently been acknowledged (Hashikawa *et al.*, 2004; Honore *et al.*, 1996; Sylvestsky *et al.*, 2002). GGS can cause a spectrum of illnesses, ranging from superficial, e.g. pharyngitis and erysipelas, to deep severe infections, e.g. necrotizing fasciitis and sepsis. Although being a less

common cause of disease, the clinical manifestations are sometimes shared with the better-known group A streptococci (GAS; *Streptococcus pyogenes*) (Cunningham, 2000). GGS infections may be less severe and also mainly found in elderly and immunocompromised individuals (Bowler *et al.*, 2001; Lewthwaite *et al.*, 2002; Vartian *et al.*, 1985).

Surface-associated proteins are important for streptococcal virulence (Navarre & Schneewind, 1999). Clinical isolates of pyogenic streptococci express M/M-like proteins on their surface (Simpson *et al.*, 1987). These coiled-coil proteins are highly homologous in structure and confer resistance to phagocytic killing, but often differ in primary sequence and ability to bind plasma proteins (Cunningham, 2000; Fischetti, 1989; Oster & Bisno, 2000). We recently identified the M-like fibrinogen-binding protein of GGS, denoted FOG (Johansson *et al.*, 2004). Besides binding fibrinogen, protein FOG mediates GGS binding to

Abbreviations: AMPs, antimicrobial peptides; BSA, bovine serum albumin; FOG, fibrinogen-binding protein of GGS; GAS, group A streptococci; GGS, group G streptococci; HSA, human serum albumin; IFN, interferon; MIG, monokine induced by interferon γ .

albumin, immunoglobulin G and collagen type I. Protein FOG acts as an adhesin and inhibits neutrophil function (Johansson *et al.*, 2004; Nitsche *et al.*, 2006). Bacterial release of M/M-like proteins could enable the bacteria to corrupt host defence functions distant from the bacterial surface and the primary site of infection. Fragments of M1 protein are released from the GAS surface by endogenous proteolytic cleavage as well as by released proteases of neutrophils (Berge & Björck, 1995; Herwald *et al.*, 2004). Such release has so far not been shown for M/M-like proteins of GGS.

Serious conditions with GGS and GAS are often preceded by milder superficial infections (Auckenthaler *et al.*, 1983). The skin and mucosa comprises a number of cell types with defence tasks such as synthesis and release of antimicrobial peptides (AMPs). The interferon (IFN) γ -governed CXC chemokine MIG/CXCL9 is a lymphocyte chemoattractant with antibacterial properties (Cole *et al.*, 2001; Liao *et al.*, 1995). MIG/CXCL9 is expressed in high amounts by inflamed epithelium, as seen in the case of streptococcal tonsillitis (Egesten *et al.*, 2007) and psoriasis (Goebeler *et al.*, 1998). The electrolyte compositions of body fluids prone to streptococcal infection differ, and may influence the activity of AMPs (Goldman *et al.*, 1997). Since GGS are generally regarded as commensals and GAS as being highly virulent, the interplay between the ionic strength of the environment and innate immunity may affect the potential of these bacteria to survive as commensals on epithelial surfaces.

In this study we address the question of the innate immune response and its effect on GGS, by investigating FOG-provoked MIG/CXCL9 expression from a monocytic cell line and the effect of a MIG/CXCL9-derived peptide on bacterial survival. By including electrolyte concentrations relevant to the *in vivo* situation in MIG/CXCL9 bactericidal assays, we reveal properties that can at least partly explain the persistence of GGS and their success as commensals.

METHODS

Bacterial strains and growth conditions. The origins of the GGS clinical isolates (G41, G10, G11, G148) have been described elsewhere (Nitsche *et al.*, 2006). The GAS strains AP1 (40/58), AP22 (59/50), AP41 (71/50) and AP49 (1/64) of serotypes M1, M22, M41 and M49, respectively, were from the World Health Organization Collaborating Centre for Reference and Research on Streptococci, Prague, Czech Republic. Streptococci were routinely grown in Todd-Hewitt (TH) liquid medium at 5% CO₂ and 37 °C. Strain G45 and isogenic mutants thereof were grown in the presence of erythromycin at a final concentration of 1 µg ml⁻¹, to maintain a knock-out phenotype (Nitsche-Schmitz *et al.*, 2007). For heat-killing of bacteria, a 2 × 10⁹ ml⁻¹ bacterial suspension was placed at 80 °C for 20 min, collected by centrifugation and resuspended in sterile-filtered RPMI 1640 (Gibco) with bovine or human serum albumin (BSA or HSA respectively); 2 × 10⁸ c.f.u. were added to individual wells.

Chemokine-producing cells. The human monocytic cell line THP-1 (ATCC #TIB-202) was routinely grown at 37 °C in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum

and 50 µg gentamicin ml⁻¹ (all from Gibco). For experiments, cells were washed once in RPMI 1640 and resuspended in RPMI 1640 containing 0.4 or 4 mg HSA ml⁻¹, or 0.4, 4 or 6 mg BSA ml⁻¹. Experiments were performed using 0.5 × 10⁶ cells ml⁻¹ in cell culture plates (Nunc). The human pharyngeal epithelial cell line Detroit 562 (ATCC #CCL-138) was cultured in Minimal Essential Medium (MEM) with Earle's salts (Invitrogen Life Technologies) supplemented with 2 mM glutamine (Invitrogen), 10% heat-inactivated fetal bovine serum (Invitrogen), 0.25 µg amphotericin B ml⁻¹ (Fungizone; Invitrogen), penicillin/streptomycin (PEST) (100 U ml⁻¹ and 0.1 ng ml⁻¹ respectively; Invitrogen). Detroit 562 cells were used in experiments when close to confluence in 6 mg BSA ml⁻¹ in MEM. For both cell types, MIG/CXCL9 was induced by IFN γ (1000 U ml⁻¹) where appropriate.

Proteins, labelling and bacterial binding assay. Recombinant proteins FOG and M1 were purified as described previously (Åkesson *et al.*, 1994; Johansson *et al.*, 2004) with the exception that protein FOG and constructs thereof lack the GST-tag used for purification. Endotoxin levels varied between 100 and 250 EU ml⁻¹, i.e. 2 ng ml⁻¹ at most, as assessed using the *Limulus amoeba* lysate technique (a kind service of Dr L. Larsson, Clinical Microbiology, Lund University). Preparations used were more than 95% pure as judged by SDS-PAGE. FITC-labelling of proteins (EZ-labelling, Pierce) was carried out in accordance with the manufacturer's recommendations. Human IgG and fibrinogen (Sigma) were labelled with ¹²⁵I using Iodobeads (Pierce). HSA was labelled using the Bolton-Hunter reagent. Assessment of bacterial binding of labelled proteins has been described (Björck & Kronvall, 1984).

Surface release of FOG, SDS-PAGE and blotting methods. Bacterial overnight culture supernatants from the FOG-expressing strain G41 were recovered by centrifugation, sterile-filtered and precipitated with 5% trichloroacetic acid (Merck). Alternatively, G41 was cultured to mid-exponential phase, washed once and then, in a total volume of 0.5 ml PBS (2.7 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl, 8.1 mM Na₂HPO₄), incubated with 2.5 mU human neutrophil elastase (Sigma) at 37 °C for 30 min. Strain G148, lacking *fog*, was used as a control. Bacteria were removed by centrifugation; samples were resolved in SDS loading buffer and then loaded in duplicate onto a 10% (w/v) acrylamide SDS-PAGE gel. Proteins were separated under reducing conditions. Eurosequencing carried out NH₂-terminal sequencing of proteins excised from acrylamide gels. For blotting, proteins were electroblotted onto a PVDF membrane, which was incubated in block buffer (1%, w/v, BSA, 0.05% Tween 20 in PBS) for 3 × 20 min. To detect protein FOG, membranes were incubated for 3 h at room temperature with unlabelled fibrinogen (50 µg ml⁻¹ in 1%, w/v, BSA in TBST; 0.05 M Tris, 0.15 M NaCl, 0.05%, v/v, Tween 20 pH 7.5) and probed with a rabbit anti-fibrinogen antibody (diluted 1:3000, DAKO). A secondary horse-radish-peroxidase-conjugated antibody (dilution 1:3000, Bio-Rad) raised in goat was used to detect bound primary antibody. To confirm that the antibodies were not interacting with membrane-bound proteins in a non-immune fashion, the membrane was incubated with radiolabelled fibrinogen in parallel, as a control. In both cases of detection the membrane was washed and developed using an imaging system (Fuji).

Absorption experiments and chemokine response. In evaluation of the chemokine response, equimolar concentrations (0.14 × 10⁻⁹ mol) of proteins FOG, FOG 1-C and M1 were used. The amount used correlated with the maximum MIG/CXCL9 response by M1 in THP-1 cells. In parallel, an LPS concentration of 2 ng ml⁻¹, corresponding to the maximum levels found in the recombinant protein preparations, was used. Cell supernatants were analysed for MIG/CXCL9 by ELISA according to the manufacturer's instructions (R&D Systems). In a separate set of experiments, G45 and isogenic mutants thereof were

added to THP-1 cells in medium containing 0.4 or 4 mg HAS ml⁻¹, or 0.4, 4 or 6 mg BSA ml⁻¹). Control wells were treated with IFN γ alone. Incubation proceeded at 37 °C for 24 h and supernatants were analysed for MIG/CXCL9 content by ELISA.

Strain G45 and the isogenic mutants thereof (2×10^8 c.f.u. in pellet) were incubated in 100 % saliva, 100 % plasma or HSA solution (4 mg ml⁻¹ in PBS) at room temperature for 1 h, washed extensively in PBS, and bound proteins eluted using 0.2 M glycine pH 2. Eluates were separated by SDS-PAGE under reducing conditions and visualized by Coomassie brilliant blue staining.

Real-time PCR. Total RNA was prepared from THP-1 cells using the Trizol reagent according to the manufacturer's instructions (Invitrogen Life Technologies). Real-time PCR was performed in triplicates of each sample on a Bio-Rad IQ 5 cycler after reverse transcriptase conversion of 50 ng total RNA to cDNA (TaqMan). The SYBRgreen mastermix and the housekeeping GAPDH gene were from Bio-Rad and the labelled MIG/CXCL9 primers were purchased from Applied Biosystems.

Flow cytometry analysis of protein binding. THP-1 cells were incubated with equimolar concentrations (9.13 μ M) of FITC-labelled proteins for 1 h at 37 °C. Following a wash step in PBS, samples were analysed on a FACSCalibur flow cytometer (Becton-Dickinson). FITC-labelled BSA was first related to autofluorescence and then used as a control for cells incubated with protein FOG or M1.

Bactericidal assays. Bacterial overnight cultures in TH were reinoculated in fresh medium and further cultured to mid-exponential phase. After washing three times with sterile 10 mM Tris/HCl, pH 7.5, supplemented with 5 mM glucose, bacterial numbers were adjusted to 2×10^9 c.f.u. ml⁻¹ using an optical density standard curve. Dilutions were made in buffer containing 0, 5, 50, 100 or 150 mM NaCl. Approximately 1×10^5 c.f.u. in 50 μ l were then incubated, with or without increasing amounts of protein MIG/CXCL9 (Peprotech) or the MIG/CXCL9-derived synthetic peptide SAD-27 (amino acid residues 79–105 of the holoprotein), for up to 1 h at 37 °C. Suspensions were diluted in the respective buffers and plated on TH solid medium. C.f.u. were counted following an overnight incubation at 37 °C and the results expressed as percentage killing as compared to bacteria incubated in the respective buffers alone.

Electrostatic potential surface modelling. Modelling of the electrostatic potential surface of MIG/CXCL9 was carried out using the DelPhi software (http://wiki.c2b2.columbia.edu/honiglab_public/index.php/Software) with the following parameters: the nonlinear Poisson–Boltzmann equation $\epsilon_R(\epsilon_i = \epsilon_o) = 80$ at +4 kT and –4 kT. $C_{salt} = 0$ and 150 mM were investigated. Results were depicted in blue surrounding the structural MIG/CXCL9 model reported previously (Egsten *et al.*, 2007).

RESULTS

Both GGS and GAS enhance MIG/CXCL9 expression

To investigate possible differences in innate immune response, GAS and GGS bacteria were added to THP-1 cells (a human monocytic cell line) and to Detroit 562 cells (an epithelial cell line) for 24 h and expression of the chemokine MIG/CXCL9 was measured (Fig. 1a). In contrast to GAS, GGS multiply rapidly in cell culture medium and to standardize the number of c.f.u. added to

the monocytes, heat-killed bacteria were used. The number of c.f.u. added to the cells was monitored by parallel plating of live bacteria on solid TH medium and did not diverge significantly from the number calculated from a standard curve (data not shown). Moreover, binding of ligands such as albumin, IgG and fibrinogen to GGS and GAS were not affected by heat-killing. A dose–response curve of heat killed bacteria showed a maximum MIG/CXCL9 release using 2×10^8 c.f.u. (data not shown). For THP-1 cells, the presence of bacteria elicited an overall high MIG/CXCL9 response, with levels ranging from 102 500 pg ml⁻¹ (strain G148) to 275 100 pg ml⁻¹ (strain AP1) (Fig. 1a). No significant difference in induction levels was observed between GAS (mean $203\,247 \pm 60\,353$ pg ml⁻¹) and GGS (mean $173\,000 \pm 47\,576$ pg ml⁻¹) strains. This indicates that there is no difference between the species with respect to innate immune recognition by THP-1 cells. The lower response comparing strain G148 (which naturally lacks protein FOG) to strain G41 (178 700 pg MIG/CXCL9 ml⁻¹) indicated a role for protein FOG in elicitation of a MIG/CXCL9 response. For the epithelial cell line, levels of MIG/CXCL9 were threefold lower than for THP-1 and ranged from 15 500 pg ml⁻¹ (strain G148) to 72 250 pg ml⁻¹ (strain AP49). As for THP-1, GAS strains and GGS strains were equally potent inducers of MIG/CXCL9.

Protein FOG is released from the bacterial surface and binds to THP-1 cells

Next, we examined the potential of protein FOG as a stimulant of monocytes by using the monocytic cell line THP-1. To investigate whether protein FOG, analogous to M proteins of GAS (Berge & Björck, 1995; Herwald *et al.*, 2004), is released by host proteases, the FOG-expressing strain G41 was incubated with purified human neutrophil elastase. Cleavage products were analysed by SDS-PAGE and Western blots. For detection of protein FOG, antibodies directed against human fibrinogen were used, as shown in Fig. 2(a). A fragment with an apparent size of 52 kDa was released from the bacterial surface (Fig. 2a, lane 2). A fragment smaller than the full-length protein FOG (Fig. 2a, lane 1), was also found in TCA-precipitated culture medium obtained from the early stationary phase of bacterial growth (Fig. 2a, lane 3). To avoid false-positive results through non-immune binding of the detection antibodies to e.g. protein G, radiolabelled fibrinogen was also used. These experiments confirmed the results of the previous experiments using antibodies. As the isogenic FOG mutant G45 does not bind fibrinogen (Nitsche-Schmitz *et al.*, 2007), we could exclude other fibrinogen-binding proteins on FOG-expressing bacteria. Strain G148, which naturally lacks protein FOG, was negative in both supernatant sample and following elastase incubation (data not shown). NH₂-sequencing of the 52 kDa protein band showed 100 % identity to the NH₂-terminus of the full-length protein FOG. By size calculation we could conclude that processing most likely occurs at the C-terminal region between amino acid residues 472 and 520, generating a

Fig. 1. GGS and GAS enhance synthesis of MIG/CXCL9 in monocytic and epithelial cells. Surface-bound protein FOG contributes to an innate immune response, which is not altered by bacterial albumin binding. (a) THP-1 (black bars) and Detroit 562 (white bars) cells were stimulated with heat-killed bacteria of the GGS strains G10, G11, G41 and G148, or the GAS strains AP1, AP22, AP41 and AP49 for 24 h. Secretion of MIG/CXCL9 was measured by ELISA; means $\pm$ SD of at least three experiments are shown. (b) Heat-killed bacteria were added to THP-1 cells in media containing 0.4 mg ml⁻¹ (light grey bars) or 4 mg ml⁻¹ (dark grey bars) HSA. After 24 h, supernatants were analysed for MIG/CXCL9 content by ELISA. Means $\pm$ SD of three experiments are shown. Results were compared using the paired Student's *t*-test and *P* < 0.05 was considered significant. (c) A binding assay using radiolabelled HSA and the bacterial surface as solid phase showed protein G as the primary interaction partner for HSA. Black diamonds, G45; dark grey squares, G45ΔFOG; light grey triangles, G45ΔpG.

fragment that corresponds to the recombinant construct FOG1-C (aa 1–493; see schematic representation in Fig. 2a; Johansson *et al.*, 2004). The presence of soluble protein FOG in the context of a GGS infection hence seems plausible and motivated the use of FOG 1-C in this study.

We have previously described that protein FOG interferes with the function of human neutrophils (Johansson *et al.*, 2004), and to examine a possible interaction with other cells of the immune system we analysed binding of FOG1-C to THP-1 cells by flow cytometry (Fig. 2b). To investigate potential differences in innate immune response in response to GGS and GAS M proteins, we included protein M1, a well-studied M protein of GAS. As a control for background binding of unspecific labelling compound we used FITC-BSA, with a background binding of 6.8 % as compared to autofluorescence. Monocytes incubated with the FITC-labelled proteins FOG or M1 demonstrated a clear binding of the proteins as compared to cells labelled with FITC-BSA. The relative numbers of cells binding the two M proteins were similar (24.7 % and 27.6 % for M1 and FOG1-C, respectively). Addition of IFN γ did not affect

binding of the investigated proteins (data not shown), indicating that the cell surface receptor is not affected by the presence or absence of IFN γ .

Protein FOG induces an increase of MIG/CXCL9 expression

To examine a possible functional response after cellular binding of proteins FOG and M1, production of MIG/CXCL9 by THP-1 cells was investigated using these proteins as stimulants. In the presence of IFN γ , soluble FOG1-C and M1 triggered a time-dependent increase in expression of MIG/CXCL9 as determined by ELISA. However, using equimolar concentrations of the proteins, FOG1-C failed to generate chemokine production to the same level as M1, which was a potent inducer (Fig. 3a). After 3 h, the FOG1-C-stimulated cells showed a lower MIG/CXCL9 response than cells stimulated with M1 (1.3-fold compared to 3-fold for FOG1-C and M1, respectively, as compared to the IFN γ -treated control). At 18 h of stimulation M1 protein generated approximately twice the

Fig. 2. Protein FOG is released from GGS and binds to monocytic cells. (a) Protein FOG (recombinant full-length as control, lane 1) was found in the bacterial supernatant of G41 incubated for 30 min at 37 °C with neutrophil elastase (lane 2). Lane 3 shows TCA-precipitated G41 growth medium. Presence of protein FOG was detected by SDS-PAGE followed by blotting. The membrane was incubated with unlabelled human fibrinogen followed by a peroxidase-conjugated goat antibody against fibrinogen. The NH₂-terminal sequence (indicated by a horizontal arrow) corresponded to the NH₂-terminus of protein FOG, demonstrating a C-terminal processing. A schematic representation of protein FOG is shown, where the designations A, B, S, C and D refer to the domain nomenclature of other known M proteins. The vertical arrow points to the probable elastase cleavage site. (b) THP-1 cells were incubated with equimolar concentrations (9.13 µM) of FITC-labelled proteins BSA, FOG1-C or M1 for 1 h at 37 °C. Binding levels of proteins FOG and M1, assessed by flow cytometry, are compared to the level of FITC-BSA bound to the cells. The FITC-BSA level is compared to autofluorescence.

amount of MIG/CXCL9 as did FOG 1-C (Fig. 3a). These results were confirmed by real-time PCR, showing a number of MIG/CXCL9 transcripts at 3 and 18 h of stimulation corresponding to experiments analysed by ELISA (Fig. 3b). Inter-experimental variations motivated the depiction as fold increase of MIG/CXCL9 release (ELISA) and the showing of one representative real-time PCR experiment ($n=3$). LPS controls excluded significant effects from the low levels of contaminating endotoxin in the protein preparations (not shown). As in the case of THP-1 cells, protein FOG (1861 ± 127.1 pg ml⁻¹) elicited a significantly lower MIG expression as compared to protein M1 ($13\,625 \pm 1515$ pg ml⁻¹) in Detroit 562 cells.

Protein G is a surface protein abundantly expressed among GGS strains, and binds IgG and albumin with high affinity (Björck & Kronvall, 1984; Björck *et al.*, 1987). To further validate the contribution of protein FOG to MIG/CXCL9 expression, the GGS strain G45 (wild-type) and the isogenic mutants G45ΔpG (devoid of protein G) and G45ΔFOG (devoid of protein FOG) were tested for their ability to induce MIG/CXCL9 expression. Similar amounts of MIG/CXCL9 were induced by G45ΔpG and the wild-type strain G45 (Fig. 1b). In contrast, G45ΔFOG gave rise

to a significantly reduced expression of MIG/CXCL9, demonstrating that also at the bacterial surface protein FOG contributes to MIG/CXCL9 induction. Stimulation of THP-1 cells is routinely performed in the presence of BSA (6 mg ml⁻¹). Due to the affinity of GGS for HSA, the expression of MIG/CXCL9 was also investigated in the presence of HSA at concentrations of physiological relevance at sites of infection (0.4 and 4 mg ml⁻¹). In all experiments, significantly decreased levels of MIG/CXCL9 were obtained with G45ΔFOG, while G45ΔpG and wild-type G45 were equally potent MIG/CXCL9 inducers.

In parallel, binding experiments using radiolabelled HSA demonstrated that at the bacterial surface protein G serves as the main albumin-recruiting protein (Fig. 1c). Recruitment of albumin to the surface of G45 and the isogenic mutants, from human plasma, saliva and an albumin solution serving as control, further emphasized this finding (data not shown). Taken together, the results demonstrate that on the surface of GGS, protein FOG, but not protein G, clearly contributes to innate immune recognition and a subsequent innate immune response. Additionally, the recruitment of HSA or BSA from the surrounding environment has a limited impact on this result.

Fig. 3. Proteins FOG and M1 enhance synthesis of MIG/CXCL9 in monocytes. THP-1 cells were stimulated with equimolar amounts of FOG1-C (grey bars) or M1 (black bars) in the presence of 1000 U IFN γ ml $^{-1}$, for 3 and 18 h at 37 °C. Cells stimulated with IFN γ alone served as control (white bars). (a) Cell supernatants were analysed for MIG/CXCL9 content by ELISA and the results expressed as fold increase compared to the control. Means $\pm$ SD for these experiments ($n=3$) are shown. (b) Real-time PCR showing the number of MIG/CXCL9 transcripts after 3 and 18 h of stimulation with FOG1-C or M1. The means $\pm$ SD for one representative experiment are shown. Each experiment was performed in triplicate.

GGs show tolerance to the antimicrobial effect of MIG/CXCL9 and the MIG/CXCL9-derived peptide SAD-27

Having established that both GGS and GAS cause release of MIG/CXCL9 from monocytes, we proceeded to investigate the susceptibility of these species to the antibacterial activity of MIG/CXCL9. The fact that GGS can exist as part of the normal flora, whereas GAS more often cause disease, raised the question whether commensal bacteria are more resistant to AMPs. We conducted a number of bactericidal assays using MIG/CXCL9 on strains G41 and AP1. In parallel we tested the synthetic MIG/CXCL9-derived peptide SAD-27, based on the antibacterial part of the MIG/CXCL9 molecule (Egsten *et al.*, 2007). As the effect of many AMPs is reduced by increasing ionic strength (Bals *et al.*, 1998; Goldman *et al.*, 1997; Yang *et al.*, 2003) we also investigated if the killing by MIG/CXCL9 or SAD-27 would be altered by NaCl at the concentrations found in unstimulated saliva (5 mM) and sweat (50 mM).

Strains G41 and AP1 were killed in a dose-dependent manner, with close to 100 % killing at 0.09 μ M holoprotein

MIG/CXCL9 (Fig. 4a). The same levels of bacterial killing were reached by 0.66 μ M of the synthetic peptide SAD-27 (Fig. 4b). Although a higher concentration of the peptide was required for bacterial killing, no additive or stabilizing effect was noted when using the holoprotein. In the presence of 50 mM NaCl, G41 bacteria were resistant to the bactericidal activity of MIG/CXCL9, while 35 % of AP1 bacteria were still killed by the chemokine (Fig. 4a). Similarly the bactericidal activity of SAD-27 was inhibited in the presence of NaCl (Fig. 4b).

Kinetics of the SAD-27-mediated killing at 0.66 μ M demonstrated that both G41 and AP1 bacteria were killed already after 20 min (Fig. 4c). The addition of 50 mM NaCl delayed killing of both strains by 5 min and reduced the level of killing of AP1 to 40 % after 30 min. For G41 the presence of NaCl had a greater negative impact on the bactericidal effect of SAD-27-mediated killing, as the maximum level of killing after 1 h was only 17 %.

Next, we investigated the sensitivity of GGS and GAS to ion concentrations corresponding to the levels in body compartments prone to streptococcal infection: saliva (5 mM), sweat (50 and 100 mM), and plasma (150 mM) (van der Merwe *et al.*, 2002). We found that G41 was more tolerant than AP1 to concentrations exceeding 5 mM NaCl (data not shown). At 50 mM, the reduction of c.f.u. (i.e. killing) after 1 h was markedly higher for the AP1 strain as compared to G41 bacteria. At most, G41 showed a decline in c.f.u. of 40 % whereas the AP1 decline approached 80 %. A similar difference in NaCl sensitivity was obtained with other tested isolates of GGS and GAS. In summary, the results suggest that GGS strains are more tolerant than GAS to physiological NaCl concentrations found at sites relevant to streptococcal colonization and infestation, and also more resistant to the bactericidal activity of MIG/CXCL9 expressed at these sites.

Influence of salt on the electrostatic surface potential of MIG/CXCL9

Finally, the effects of increasing ionic strength on the electrostatic surface properties of MIG/CXCL9 were examined. Modelling of the electrostatic surface potential of MIG/CXCL9 revealed a greatly diminished field surrounding the NH $_2$ -terminus at 150 mM NaCl compared with absence of salt (Fig. 4d). The C-terminal surface potential was moderately decreased, indicating a retained positive net charge of the bactericidal α -helical domain. These results demonstrate that although 150 mM NaCl reduces the electrostatic surface potential of the α -helical domain of MIG/CXCL9, the bactericidal activity is likely to be retained. Thus, the differences observed in MIG/CXCL9 killing of GGS and GAS point to differences in biophysical properties of their respective surfaces. Further investigations may explain these findings.

Fig. 4. NaCl alters the MIG/CXCL9 surface potential and reduces MIG/CXCL9-mediated killing of GGS. The streptococcal strains G41 (open symbols) and AP1 (solid symbols) (2×10^5) were incubated for 1 h at 37 °C with different concentration of (a) MIG/CXCL9 and (b) the synthetic peptide SAD-27 in the absence (squares) or presence (circles) of 50 mM NaCl. Following incubation, appropriate dilutions were plated onto TH agar. Plates were incubated at 37 °C overnight and the number of c.f.u. was determined. (c) Killing kinetics of test strains G41 and AP1 using SAD-27 in the absence or presence of 50 mM NaCl. Bacterial counts were assessed after an overnight incubation at 37 °C and expressed as a percentage of the control. (d) Modelling of the electrostatic potential surface in the absence and presence of 150 mM NaCl, conducted using the software DelPhi and overlaid on the MIG/CXCL9 structural cartoon.

DISCUSSION

Although GGS recently have been acknowledged to cause both superficial and invasive disease, they have traditionally been regarded as commensals. Here we demonstrate the ability of the recently discovered protein FOG (Johansson *et al.*, 2004) to provoke an innate immune response. During an encounter between neutrophils and FOG-expressing bacteria, neutrophil elastase can release a fragment of protein FOG with retained biological activity. This suggests that also *in vivo* it is plausible that M/M-like proteins from GGS are cleaved from the bacterial surface, eliciting responses from surrounding immune cells. In the current study we demonstrate that protein FOG1-C, a fragment relevant in size to that found after GGS encounter with neutrophils or directly released from the bacterial surface, binds to monocytes and together with IFN γ triggers production of the chemokine MIG/CXCL9. Soluble protein FOG differed markedly from the classical virulence factor protein M1 of GAS in inducing production of the chemokine MIG/CXCL9. At equimolar amounts, M1 caused a more pronounced release of MIG/CXCL9 after 18 h. Analysis of gene expression of MIG/CXCL9

confirmed these findings. The less potent induction of MIG/CXCL9 expression by protein FOG may, in an infection, signify a reduced recruitment of T-cells to the site. This may result in a general reduction of inflammatory mediators originating from these cells. Infections with GAS, but traditionally not GGS, are often associated with an exaggerated immune response resulting in tissue damage and severe disease in the host. Our results may signify GGS's ability to act as a commensal and avoid causing a robust immune response.

When stimulating a monocytic cell line with heat-killed whole bacteria, G41 and G148 also induced a lower expression of MIG/CXCL9 as compared to AP1, although no significant difference between GGS and GAS was observed when additional strains were analysed. The same was found for stimulation of Detroit 562 cells. However, for THP-1, the markedly lower MIG/CXCL9 level induced by G148 compared to that of G41 led to the proposition of a role for protein FOG. The plausible role for protein FOG was confirmed using an isogenic mutant lacking protein FOG. Recent testing of isogenic mutants of GAS strain AP1 in our group, to study the contribution of M1 to MIG/

CXCL9 expression, found no differences between the wild-type AP1 strain and mutants lacking protein M1 (Eliasson *et al.*, 2007). Seemingly, when anchored to the bacterial surface protein FOG adds to the expression of MIG/CXCL9, whereas in the soluble form it is only a moderate inducer. In addition, the results show that although protein G is the primary albumin-recruiting surface protein of GGS, neither this protein nor albumin binding contributes to an innate immune response and the subsequent chemokine expression in the cell type investigated here.

Cell walls of Gram-positive bacteria are known to cause chemokine release from monocytes (Heumann *et al.*, 1994) and such a release of TNF α , IL-1 and IL-6 was suggested to contribute to the severity of bacterial septicemia (Whicher & Evans, 1990). Differential host chemokine response has also been implicated as a determinant of severity in GAS infection (Norrby-Teglund *et al.*, 2000). Furthermore, significant amounts of pro-inflammatory cytokines (IL-1 α and β , TNF α , IL-6 and IL-8) were secreted from monocytes in response to heat-killed GAS (Müller-Alouf *et al.*, 1994), and these bacteria also induced transcription of the MIG/CXCL9 gene in macrophages (Veckman *et al.*, 2003). However, reports on GGS causing such chemokine release have until now been scarce. Issues concerning how commensals avoid evoking an immunoresponse are interesting and have been discussed (Dieleman *et al.*, 2004; Granucci & Ricciardi-Castagnoli, 2003). The current study adds new findings to this field.

The observation that the investigated GGS strains are more tolerant to salt concentrations typical of various body fluids suggests a structural or metabolic advantage for GGS in the quest for survival and persistence in the colonized host. In NaCl concentrations exceeding that of unstimulated saliva, MIG/CXCL9-mediated killing of GGS was reduced. Also, in concentrations corresponding to that of sweat, GGS were better survivors than GAS. GGS are known to cause severe infections in the immunocompromised host, and socio-economically challenged groups often suffer from GGS infections (Lewthwaite *et al.*, 2002; Vartian *et al.*, 1985). In sweat of malnourished individuals NaCl levels are increased (Rodrigues *et al.*, 1994) and hence malnutrition may be a risk factor for GGS colonization. The resistance to high levels of NaCl is shared by other pathogens (Schmid *et al.*, 1995).

Generally AMPs carry a positive net charge, which enables the interaction with negatively charged surfaces of bacteria. Modelling of the electrostatic surfaces of MIG/CXCL9 showed an altered, but yet intact ability of the C-terminus to exert its bactericidal effect. Plausibly, the presence of NaCl exceeding 5 mM reveals a difference in net surface charge between GGS and GAS. A higher level of lipoteichoic acid (LTA) has been reported for the cell wall of GAS compared to GGS, rendering a more negatively charged and hydrophobic surface (Miörner *et al.*, 1983). Possibly the LTA-related negative surface charge of GAS could explain the susceptibility of these bacteria to AMPs. Both the AMP killing of GAS in NaCl buffer, rather than

GGs, and the effect of NaCl alone imply differences in the architecture of the streptococcal cell wall that could greatly affect the degree of persistence during colonization. Recognition of GGS as an emerging human pathogen with distinct survival skills is therefore important.

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