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Streptococcal Surface Proteins Activate the Contact System and Control Its Antibacterial Activity*

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Background: The contact system, a branch of innate immunity, is also exploited by bacterial pathogens.
Results: Group G streptococcal surface proteins activate the system and protect bacteria against the antibacterial effect of the activation.
Conclusion: Negative effects of contact system activation are counteracted by group G streptococci.
Significance: The results define novel molecular interactions with implications for bacterial pathogenicity.

Group G streptococci (GGS) are important bacterial pathogens in humans. Here, we investigated the interactions between GGS and the contact system, a procoagulant and proinflammatory proteolytic cascade that, upon activation, also generates antibacterial peptides. Two surface proteins of GGS, protein FOG and protein G (PG), were found to bind contact system proteins. Experiments utilizing contact protein-deficient human plasma and isogenic GGS mutant strains lacking FOG or PG showed that FOG and PG both activate the procoagulant branch of the contact system. In contrast, only FOG induced cleavage of high molecular weight kininogen, generating the proinflammatory bradykinin peptide and additional high molecular weight kininogen fragments containing the antimicrobial peptide NAT-26. On the other hand, PG protected the bacteria against the antibacterial effect of NAT-26. These findings underline the significance of the contact system in innate immunity and demonstrate that GGS have evolved surface proteins to exploit and modulate its effects.

Streptococcal research and diagnosis have traditionally been focused on group A streptococci (GAS).[2] It is, however, being increasingly recognized that also group G streptococci (GGS) contribute substantially to streptococcal disease burden (1–5), and the clinical panorama for GGS is similar to that for GAS: pharyngeal and dermal colonization and infection, soft tissue infection, sepsis, and post-infection sequelae (1, 6, 7).

The contact system, or the intrinsic pathway of coagulation, consists of four proteins: the three serine proteases factor XII (FXII), FXI, and plasma kallikrein (PK) and the non-enzymatic cofactor high molecular weight kininogen (HK) (8, 9). HK, which in the bloodstream circulates in complex with FXI or PK, consists of six domains: D1–D3 are cystatin-like, D4 contains the proinflammatory bradykinin (BK) peptide, D5 binds to negatively charged surfaces, and D6 mediates binding to FXI and PK. The contact system is an inducer of coagulation on artificial negatively charged surfaces, but it also plays a role in immunity and inflammation (10–13). Activation of the system starts with the activation of FXII (FXIIa) on negatively charged surfaces (14). FXIIa then cleaves and activates FXI and PK in their complexes with HK. This has two main consequences; FXIa initiates the intrinsic pathway of coagulation, whereas the cleavage of HK by activated PK generates antimicrobial peptides and the proinflammatory BK peptide (for review, see Ref. 10).

There are various mechanisms by which streptococci modulate or prevent immune responses (15, 16). For example, SIC, a protein secreted by GAS, interferes with complement-mediated hemolysis (17) and blocks the antibacterial activity of antimicrobial peptides generated through contact activation (18). It has also been shown that SIC and SpeB, a secreted cysteine protease of GAS, have opposing effects on the contact system (19, 20). Although SpeB, through cleavage of HK, releases the proinflammatory BK peptide (20), SIC is anti-inflammatory by inhibiting the binding of HK to endothelial cells, thereby reducing contact activation (19). Previous work has shown that FOG, a GGS surface protein related to M proteins of GAS (21), and protein G (PG), another GGS surface protein (22, 23), both bind human IgG. However, a recent study demonstrated different effects of their interactions with IgG; whereas FOG-bound IgG was capable of interacting with complement factor C1q, PG-bound IgG was not (24). Thus, in contrast to FOG, PG may prevent complement recognition by the classical pathway. In this work, we investigated possible interactions of FOG and PG with the contact system. The results emphasize the intricate nature of molecular host-bacteria interactions; whereas both FOG and PG activate the procoagulant branch of the system,

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[2] The abbreviations used are: GAS, group A streptococci; GGS, group G streptococci/streptococcal; FXII, factor XII; PK, plasma kallikrein; HK, high molecular weight kininogen; BK, bradykinin; PG, protein G; HSA, human serum albumin; cfu, colony-forming units; CMK, chloromethyl ketone; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; aPTT, activated partial thromboplastin time.

only PG protects the bacteria against the antibacterial peptides generated through the cleavage of HK induced by FOG.

EXPERIMENTAL PROCEDURES

Bacteria, Growth Conditions, Preparation, and Plasma—The GGS strains (except G45) were clinical isolates collected at the Department of Clinical Microbiology of Lund University Hospital (Lund, Sweden). They were blood (G6), joint (G11), wound (G26, G36, G55, and G60), and throat (G41, G67, and G148) isolates. G45WT, G45ΔFOG, and G45ΔPG are wild-type G45 and isogenic mutants lacking FOG and PG, respectively. G45ΔFOG does not bind fibrinogen (24), indicating that it does indeed lack FOG, and G45ΔPG bound almost no human serum albumin (HSA) (data not shown). The trace binding of HSA is probably mediated by FOG. The G45WT strain was collected at the Royal Brisbane Hospital (Brisbane, Australia) from a throat infection. The bacteria were grown in 37 °C and 5% CO_2 in THY (Todd-Hewitt broth (Difco) with 0.5% yeast extract (Oxoid)). For mutants, erythromycin at a final concentration of 1 μg/ml was added. Logarithmic phase cultures were obtained by inoculating 450 μl of an overnight culture into 10 ml of fresh medium. The bacteria were washed and diluted in PBS or 50 mM Tris-HCl (pH 7.5) with 50 μM $ZnCl_2$ for incubation with plasma, in PBST (PBS with 0.5% Tween 20; Merck Schuchardt OHG) for binding of radiolabeled proteins, or in TG buffer (10 mM Tris-HCl (pH 7.5) and 5 mM glucose) for plating and counting of colony-forming units (cfu). Bacterial concentrations for incubation were determined by A_{620}, and a standard curve was developed by plating and counting cfu. Cultures with A_{620} = 0.4–0.5 were regarded as mid-log. Citrated plasma was obtained from the blood bank at Lund University Hospital or prepared by centrifugation from blood taken with Vacutainer® (BD Biosciences) from healthy donors and kept frozen at −80 °C until used. Human FXI-deficient plasma was purchased from George King Bio-Medical, Inc. (Overland Park, KS).

Proteins, Antibodies, Reagents, and Iodination—FOG1-D (amino acids 1–557), FOG1-C (amino acids 1–493) and FOG1-B (amino acids 1–278) were purified fused to GST as described previously (24). A 35-kDa PG fragment was solubilized from the bacterial surface using papain (22), and a fragment of PG containing the IgG-binding region (17 kDa) was purchased from GE Healthcare. BSA was from Saveen & Werner AB, and HSA, polyclonal IgG, and fibrinogen were from Sigma. The human contact factors HK, FXII, FXI, and PK were purchased from Kordia. The synthetic peptides based on sequences in kininogen domain D3 were described previously (25). Antibodies against NAT-26 were raised in rabbits (26). HRP-conjugated goat anti-rabbit IgG was from Bio-Rad, and HRP-conjugated protein A was from Sigma. The FXII/PK inhibitor *H*-D-Pro-Phe-Arg chloromethyl ketone (CMK) peptide was from Bachem Feinchemikalien AG. Proteins were radiolabeled with ^{125}I using IODO-BEAD® iodination reagent (Pierce) as described by the manufacturer.

Antibacterial Assay and Salvage Experiments—Bacteria grown to mid-log phase were washed and diluted to 2 × 10^6 cfu/ml in TG buffer. Fifty μl of bacterial suspension was incubated with various concentrations of NAT-26 peptide for 1 h at 37 °C. In subsequent experiments, G45ΔPG bacteria were incubated with 1.28 μM NAT-26 and various concentrations of FOG1-C and 35-kDa PG for 1 h at 37 °C. The bactericidal activity was determined by plating dilutions of the incubation mixtures on THY-agar plates, incubating them overnight at 37 °C, and counting cfu.

Slot Binding Experiments—Proteins were applied in slots to PVDF membranes (Immobilon™, Millipore) using a MilliBlot-D system (Millipore). The membranes were blocked with PBS or PBST containing BSA, incubated with ^{125}I-labeled probes diluted in PBS (or PBST) + BSA (~2 × 10^5 or 5 × 10^5 cpm/ml), and washed with PBST, and bound probes were detected with the Fuji FLA-3000 imaging system.

Bacterial Binding of Radiolabeled Proteins—Bacteria were washed and diluted to 1% (2 × 10^9 cfu/ml) in PBST, and 200 μl of bacterial solution was incubated in plastic tubes (Sarstedt) with 25 μl of premeasured ^{125}I-labeled probe (~10,000 cpm) for 30 or 60 min at room temperature. After incubation, 2 ml of PBST was added, the samples were centrifuged, and supernatants were removed. The samples were measured for γ-radiation, and the after/before incubation ratio of cpm was calculated.

Plasma Absorption and Western Blot Experiments—Mid-log bacteria were washed and diluted to 1% (2 × 10^9 cfu/ml) in PBS. Five-hundred μl of bacterial suspension was mixed with 500 μl of plasma for incubation in 50% plasma or with 400 μl of PBS and 100 μl of plasma for incubation in 10% plasma and incubated at 37 °C for 1 h with gentle mixing. The bacteria were washed three times with PBS and resuspended and incubated in 0.1 M glycine HCl (pH 2.0) for 10 min. Following centrifugation, the pH of the supernatant was adjusted to approximately neutral with 1 M Tris, followed by precipitation with 5% TCA for 30 min on ice and centrifugation at 16,000 × *g* for 30 min at 4 °C. The precipitate was resuspended in SDS running buffer and analyzed by SDS-PAGE on 4–20% gradient gels or by Tricine/SDS-PAGE (for separation of smaller proteins), followed by Western blotting on Immobilon. The membranes were blocked with skim milk (Difco) in PBST and probed with primary antibody (rabbit anti-NAT-26, 1:100 dilution) and secondary antibody (HRP-conjugated goat anti-rabbit, 1:5000 dilution) or HRP-conjugated protein A (1:5000 dilution). SuperSignal® West Pico chemiluminescent substrate (Thermo Scientific) was added, and the signal was detected with a ChemiDoc apparatus (Bio-Rad). Alternatively, bound antibodies were detected by the chemiluminescence method as described (27). PageRuler™ Plus (Fermentas) was used as a standard.

Activated Partial Thromboplastin Time (aPTT) Assays—For reconstitution of FXI-deficient plasma, mid-log G45WT, G45ΔFOG, and G45ΔPG bacteria were washed and diluted to 1% (2 × 10^9 cfu/ml) in 12.9 mM sodium citrate buffer. Five-hundred μl of bacterial suspension was centrifuged, and the pellet was resuspended in 150 μl of normal citrated plasma. Following incubation for 1 h at 37 °C under rotation, the bacteria were washed and incubated in 150 μl of FXI-deficient plasma for 30 min at 37 °C under rotation. The samples were centrifuged, and the supernatants were subjected to a standard aPTT measurement with the kaolin reagent (Dapttin®, Technoclone) as described previously (28).

Chromogenic Assay—Mid-log bacteria were washed and diluted to 10% (2×10^{10} cfu/ml) in 12.9 mM sodium citrate buffer. Thirty μl of bacterial suspension was incubated with 100 μl of citrated normal plasma with the addition of the contact system inhibitor *H*-D-Pro-Phe-Arg-CMK (final concentration of 100 μg/ml). Incubation in buffer only was used as a control. Bacteria were incubated for 1 h at 37 °C, washed with 12.9 mM sodium citrate buffer, reincubated for 30 min with 100 μl of S-2302™ (Chromogenix) chromogenic substrate, and centrifuged. A_{405} was determined for the supernatants.

Bradykinin ELISA—Mid-log bacteria were washed and diluted to 2.5% (5×10^9 cfu/ml) in 50 mM Tris-HCl (pH 7.5) supplemented with 50 μM $ZnCl_2$. One-hundred μl of bacterial suspension was incubated with 100 μl of normal human plasma for 15 min at 37 °C under rotation. After incubation, the bacteria were recovered by centrifugation at 12,000 $\times$ *g* for 3 min, the supernatant was discarded, and the bacteria were resuspended in 50 μl of buffer and incubated at room temperature for 15 min. Following centrifugation, the resulting supernatants were transferred to new tubes, and 10 μl of TCA (provided with the MARKIT-M bradykinin kit, DS Pharma Biomedical Co., Ltd.) was added. The precipitate was removed, and the supernatant was transferred to a new tube to which 50 μl of buffer B (from the bradykinin kit) was added. Samples were stored at −20 °C for further analysis. The concentration of BK in the samples was determined by ELISA as recommended by the manufacturer.

Surface Plasmon Resonance Interaction Analysis—HK was immobilized via amine coupling to a CM5 sensor chip flow chamber (GE Healthcare) at a moderate response level (*e.g.* 1500 response units). Briefly, HK was mixed with freshly prepared 100 mM *N*-hydroxysuccinimide and 400 mM *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide in equal volumes, and capping of unreacted carboxymethyl sites was achieved by a 1 M ethanolamine (pH 8) injection. A flow chamber subjected to the immobilization protocol but without any addition of protein was used as control (blank) for each experiment. FOG and PG constructs were sequentially diluted 2-fold in running buffer (10 mM HEPES, 150 mM NaCl, and 0.005% Surfactant P20 (BIAcore; pH 7.5)) and injected over the HK surface at 35 μl/min. Binding was monitored in a BIAcore 2000 instrument. Between experiments, the HK surface was strictly regenerated with multiple pulses of 2 M NaCl, followed by an extensive wash procedure using running buffer. After *x* axis and *y* axis normalization of the obtained data, the blank bulk refraction curves from the control flow chamber of each injected concentration were subtracted. Binding curves were displayed, and the association (K_a) and dissociation (K_d) rate constants were determined using BIAevaluation 4.1 software and the equation for 1:1 Langmuir binding. From these values, affinities (K_D) were calculated.

Electron Microscopy—The following samples were prepared for negative staining. G45WT, G45ΔFOG, and G45ΔPG were grown to mid-logarithmic phase, washed, and diluted to 2×10^9 cfu/ml in TG buffer. A measure of 200 μl of bacterial suspension was incubated with 2 μM NAT-26 or buffer for 1 h at 37 °C, washed with TG buffer, and finally resuspended in 200 μl of TG buffer. Complexes of HK·FOG1-C, HK·35-kDa PG, or HK·17-kDa PG were preformed by incubation of equimolar concentrations (10 nM) for 1 h at room temperature. For negative staining of bacteria and protein complexes, the samples were adsorbed to 400 mesh carbon-coated copper grids and stained with 0.75% (w/v) uranyl formate as described (29). Samples were observed in an FEI Tecnai Spirit BioTWIN transmission electron microscope (North America NanoPort, Hillsboro, OR) operated at an accelerating voltage of 60 kV. Images were recorded with an Eagle™ CCD camera.

RESULTS AND DISCUSSION

In this study, we focused on the clinical GGS isolate G45 expressing FOG and PG (G45WT) and isogenic FOG and PG deletion mutants of G45 (G45ΔFOG and G45ΔPG, respectively). PG is mainly responsible for the binding of IgG and albumin to the surface of GGS, whereas fibrinogen is bound to FOG (21, 22, 30). These binding properties were used to investigate the surface expression of the two proteins during growth. Samples of G45WT collected at early, mid-log, and stationary growth phase were subjected to a binding assay in which the bacteria were incubated with either ^{125}I-labeled fibrinogen or albumin. In all samples, the bacteria bound ~50–55% of the two added radioactive probes, demonstrating that FOG and PG were expressed during the entire growth phase (data not shown). Fig. 1 shows electron micrographs following negative staining of G45WT (Fig. 1*A*), G45ΔFOG (Fig. 1*B*), and G45ΔPG (Fig. 1*C*) from the logarithmic growth phase, where the hair-like protrusions typical of M and M-like proteins, missing in G45ΔFOG but present in G45WT and G45ΔPG, represent FOG. PG with its globular and compact structure is not visualized in these micrographs.

Next, the binding of FOG and PG to the proteins of the contact system was tested in slot binding experiments (Fig. 2*A*). The contact factors were applied to Immobilon membranes, which were probed with radiolabeled FOG or PG. FOG bound to FXII and FXI, whereas PG showed affinity for HK, PK, FXI, and FXII, *i.e.* all contact system components. When, instead, FOG and PG were applied to the membranes and radiolabeled HK was used as the probe, both proteins interacted with HK (data not shown). This kind of inconsistency, not uncommon in slot binding experiments and probably reflecting different exposure of immobilized proteins to the probe, makes it necessary to test possible protein-protein interactions with additional methods (see below). Because FXI and PK circulate in complex with HK (31, 32), the indicated binding of FXII and HK by FOG and PG suggested that both bacterial proteins can recruit the entire contact system to the bacterial surface. To further confirm and characterize the interactions between HK and the bacterial proteins (schematic representations of FOG, PG, and the fragments of the two proteins tested for HK binding are shown in Fig. 2*B*), plasmon resonance experiments were performed (Fig. 2*C*). Immobilized HK bound FOG1-D (amino acids 1–557) and FOG1-C (amino acids 1–493) with affinity constants of 6.1 and 14 nM, respectively, whereas another FOG fragment, FOG1-B (amino acids 1–278), had no affinity for HK on the BIAcore chip. The affinity constant for the interaction between 35-kDa PG and HK was 0.92 nM. In contrast, an IgG-binding fragment of PG (17 kDa) located in the C-terminal half showed no interaction with HK, suggesting that HK binding is located more C-terminally in FOG than in PG. Electron micros-

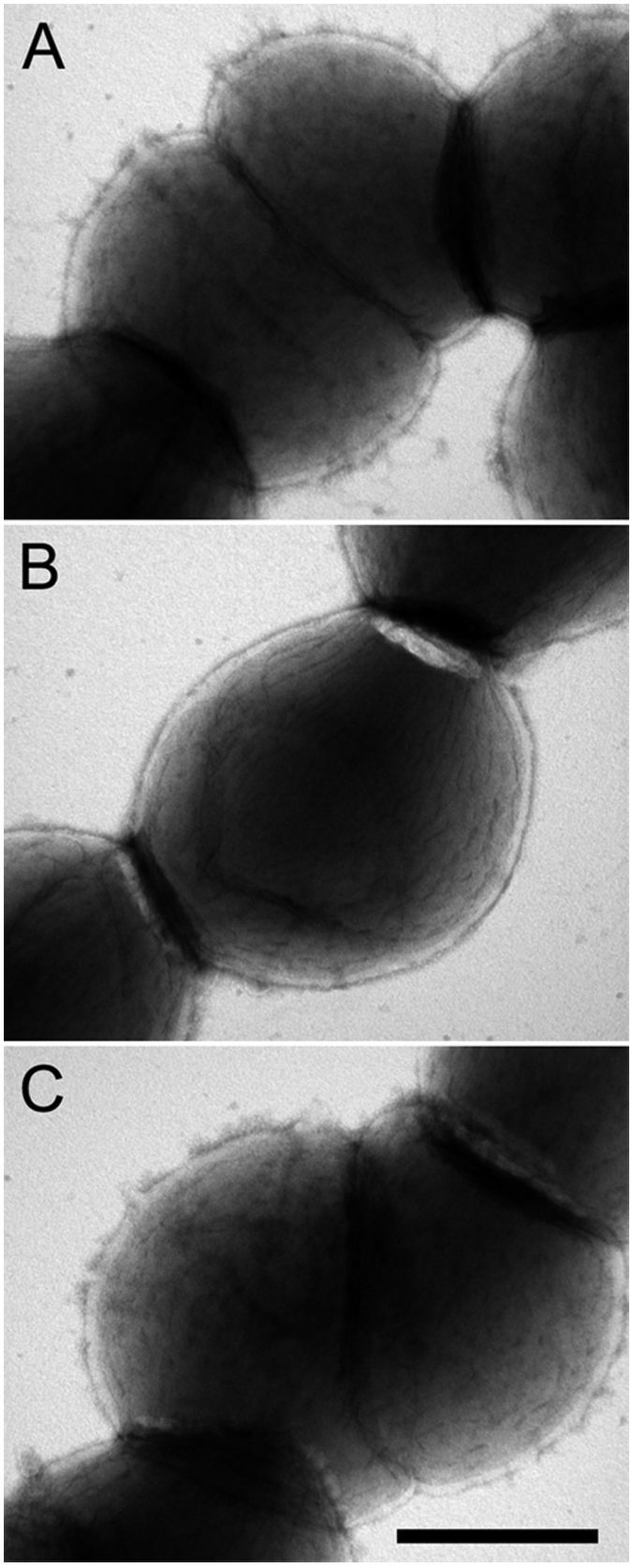

FIGURE 1. **Electron micrographs of G45WT (*A*) and mutants G45ΔFOG (*B*) and G45ΔPG (*C*) showing hair-like protrusions representing FOG in G45WT and G45ΔPG but not in G45ΔFOG.** *Scale bar* = 0.5 μm.

copy analyses following negative staining experiments were performed to visualize the proteins and the complexes between HK·FOG and HK·PG (Fig. 3). In line with the binding experiments described above, HK (*red*) was associated with one end of the rod-shaped FOG (Fig. 3*A*) and with the 35-kDa fragment of PG (Fig. 3*B*). No complexes were formed with the 17-kDa PG fragment (Fig. 3*C*). To investigate whether HK interacts also with FOG and PG at the bacterial surface, a number of clinical GGS isolates, including G45WT and its mutants G45ΔFOG and G45ΔPG, were tested for binding of ^{125}I-labeled HK (Fig. 4*A*). The results show that HK had affinity for all of the strains and that the binding of HK was reduced in the strains lacking either FOG (G45ΔFOG and G148) or PG (G45ΔPG), suggesting that HK binding is a general property of GGS and that FOG and PG are responsible for the interaction. Whether the interaction data described above, indicating a more C-terminal binding of HK to FOG, mean that HK bound to FOG is located closer to the bacterial cell wall than HK in complex with PG remains an open question. Because of its fibrous structure (Fig. 1, *A* and *C*, and Fig. 3*A*), FOG protrudes farther from the bacterial cell wall than PG.

Previous work has shown that several pathogenic bacteria such as GAS, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella*, and *Bacteroides* (28) and *Fusobacterium necrophorum* (33) bind and assemble HK and the other contact factors at their surface, leading to contact activation (for additional references, see Ref. 10). The effect of G45WT, G45ΔFOG, and G45ΔPG on the coagulant FXI-dependent branch of the contact system was investigated in an aPTT assay. In this assay, bacteria are incubated with plasma, and after centrifugation, the clotting time, induced by kaolin, is measured in the supernatant. If procoagulant contact factors are bound to and activated at the bacterial surface, the resulting effect is a combination of binding and depletion of FXI and FXII, prolonging the aPTT, and FXI activation, shortening the aPTT. To more clearly define our experimental system, FXI-deficient plasma was employed. Bacteria were first incubated in normal plasma, followed by washing and reincubation in FXI-deficient plasma, in which the aPTT was measured. In this case, a decreased aPTT will require both binding and activation of FXI, and the results in Fig. 4*B* show that G45WT and the two mutants shortened the aPTT compared with the control (FXI-deficient plasma + kaolin). These data and the experiments showing that FOG and PG bound FXI and FXII (Fig. 2*A*) suggest that both surface proteins activate the intrinsic pathway of coagulation.

When the proinflammatory branch of the contact system is activated, native HK (120 kDa) is cleaved by PK into a heavy chain (65 kDa) and a light chain (55 kDa), and the nonapeptide BK is released (9). This activation process was studied at the surface of GGS incubated with human plasma using a chromogenic assay in which the enzymatic activity of activated contact factors is determined by hydrolysis of a chromogenic substrate. Following incubation in plasma, bacteria were washed and incubated with the substrate, and the release of the chromophore was measured photometrically. Compared with G45WT, the FOG mutant exhibited reduced activity, whereas the G45ΔPG mutant, devoid of PG but expressing FOG, was not affected (Fig. 5*A*). Activation of the contact system was

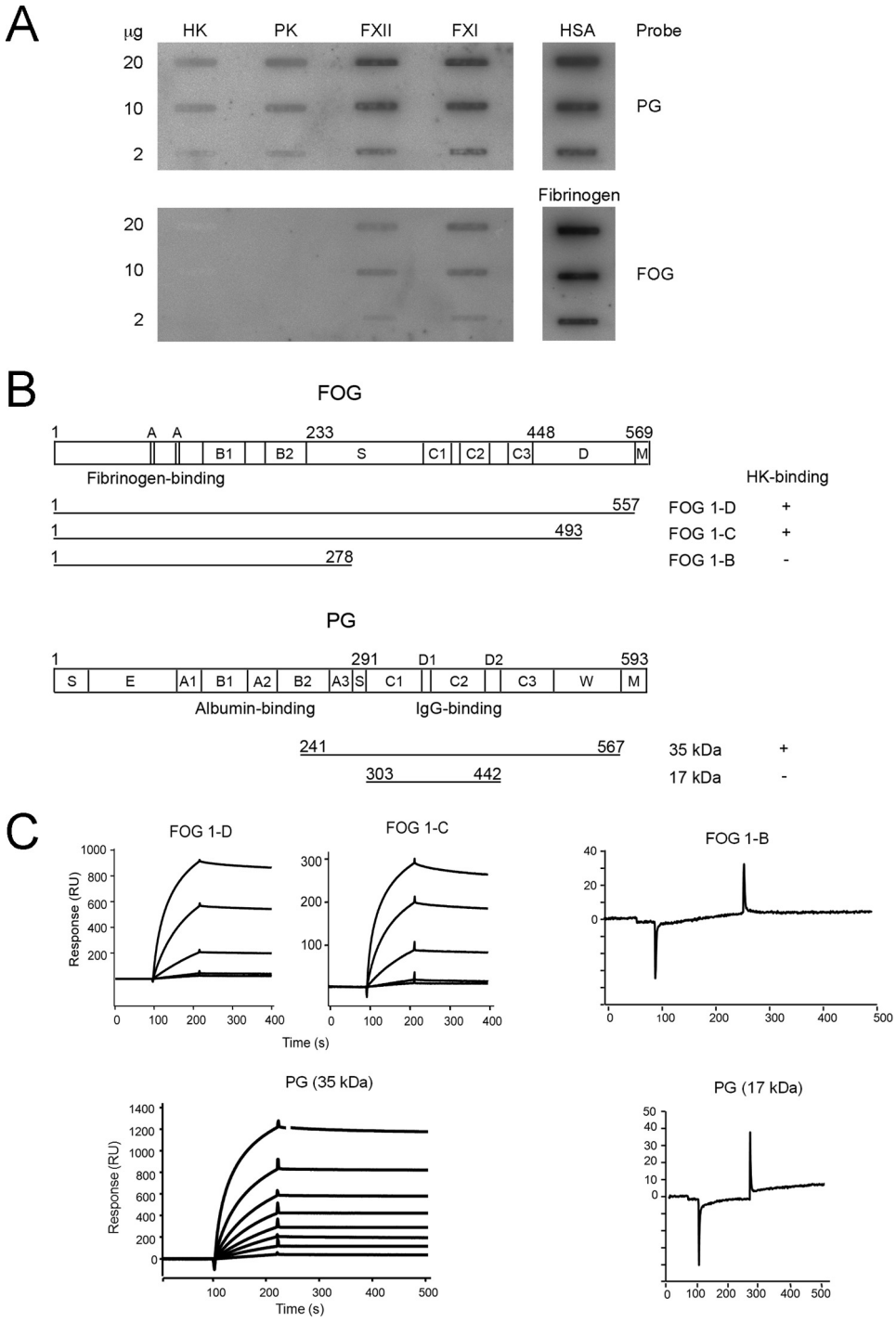

FIGURE 2. **FOG and PG interact with contact system proteins.** *A*, various amounts of contact system components were applied to PVDF membranes, which were probed with ^{125}I-labeled 35-kDa PG or FOG1-C. HSA and fibrinogen were included as positive controls for PG and FOG, respectively. *B*, schematic representations of FOG and PG. The positions of the various domains in each protein are shown, and the FOG and PG fragments used in this study are indicated. *C*, surface plasmon resonance spectroscopy experiments analyzing the interactions between HK and FOG1-D, FOG1-C, FOG1-B, and the 35- and 17-kDa PG fragments. The bacterial proteins were injected over immobilized HK, and binding was monitored in a BIAcore 2000 instrument. *RU*, response units.

blocked by the synthetic peptide *H*-D-Pro-Phe-Arg-CMK, a specific FXII/PK inhibitor (34), which was used as a control in these experiments (Fig. 5*A*). The results show that the assay indeed measures contact activation and that FOG at the bacterial surface, in contrast to PG, activates the proinflammatory branch of the system. A consequence of the enzymatic activity observed in the chromogenic assay would be the release of the proinflammatory BK peptide. Thus, G45 bacteria and the isogenic mutants were incubated in plasma, and the BK release was measured by ELISA. Although G45WT and G45ΔPG caused a substantial release of BK, the amount of BK release caused by G45ΔFOG was reduced (Fig. 5*B*). In the presence of the FXII/PK inhibitor *H*-D-Pro-Phe-Arg-CMK, the BK release was completely abolished and similar to the negative control.

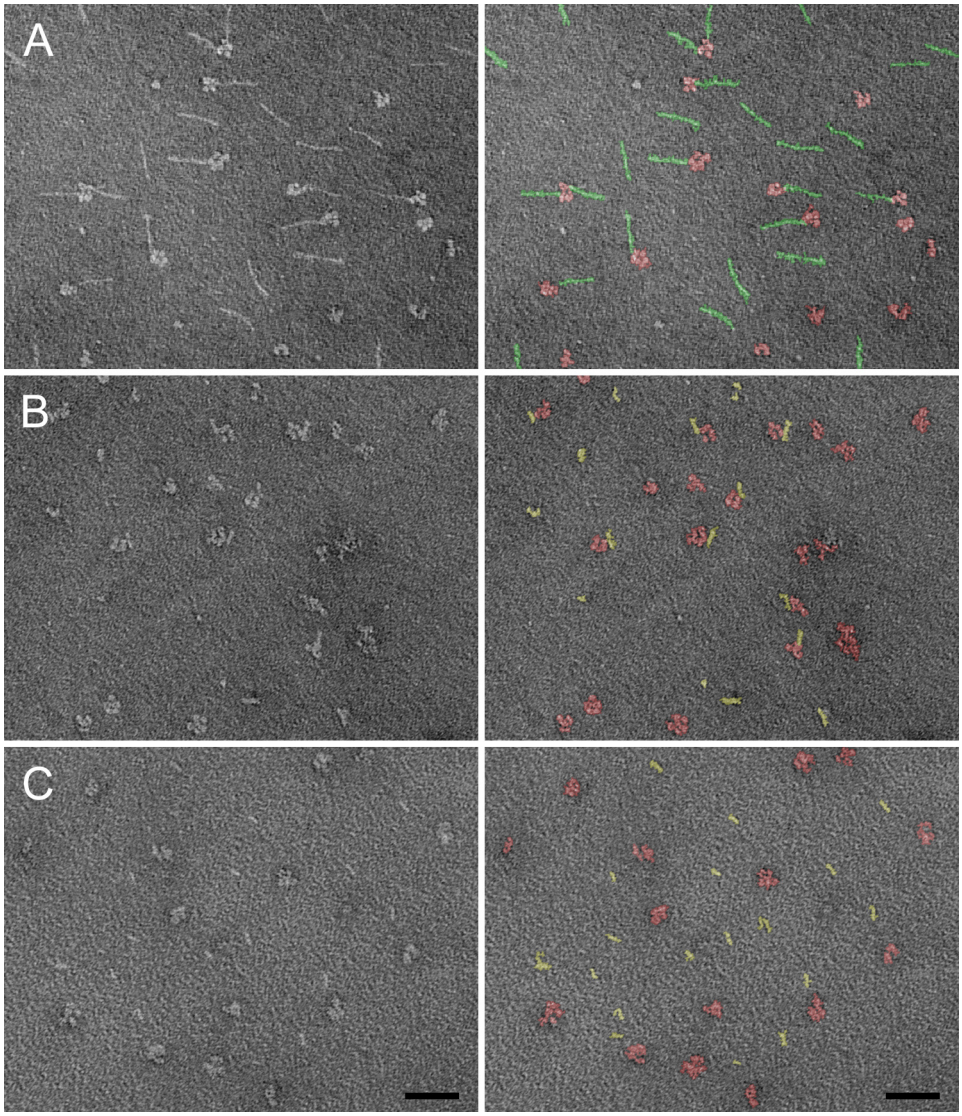

FIGURE 3. Shown are electron micrographs after negative staining of HK in complex with FOG1-C (*A*) and 35-kDa PG (*B*). HK incubated with 17-kDa PG did not form any complex (*C*). The protein complexes visualized in the *left panels* are shown in pseudocolors in the *right panels*. *Scale bars* = 50 nm.

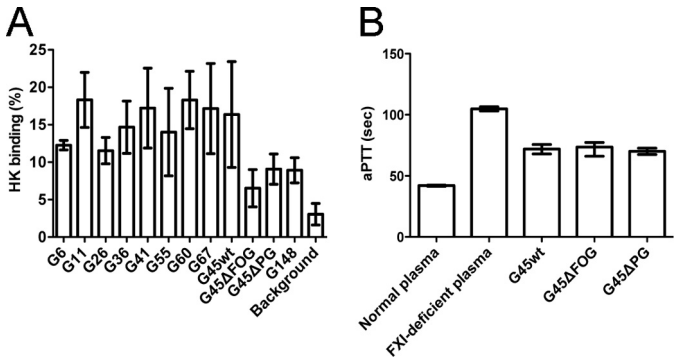

FIGURE 4. *A*, isolates of GGS bind HK. Bacteria were grown overnight and incubated with ^{125}I-labeled HK. After washing, the radioactivity of the pellets was measured. The values (percent of added radioactivity) represent the mean ± S.D. of at least three experiments. *B*, in normal plasma, FXI was adsorbed and activated by FOG and PG. Wild-type and mutant bacteria (pre-incubated with normal plasma) were added to FXI-deficient plasma to reveal the effect of FXI adsorbed to the bacterial surface. The aPTT of these plasma samples was determined. Normal plasma and FXI-deficient plasma without bacterial incubation were used as controls. Median values and the range of four experiments are indicated.

Previous work has shown that further processing of the heavy chain of HK upon contact activation generates smaller fragments, some of which contain the NAT-26 peptide from domain D3, and in contrast to intact HK, these fragments, as well as the synthetic NAT-26 peptide, are antibacterial. The antibacterial effect of NAT-26 against GAS (strain AP1) is more potent than that of LL-37 at physiological salt concentration and similar at lower salt concentrations (26). Overlapping synthetic peptides covering domain D3 were analyzed in slot binding experiments using radiolabeled FOG or PG as the probe. Both streptococcal proteins showed affinity for the NAT-26 peptide but did not interact with the other peptides (Fig. 5*D*). After incubation in 50% plasma, NAT-26-containing fragments were generated at the surface of G45WT and the two mutants as demonstrated by Western blot analyses of material eluted from the bacteria by low pH. However, the smaller NAT-26-containing fragments could not be eluted from G45ΔFOG bacteria following plasma incubation (Fig. 5*C*). In 10% plasma, which may be more representative of inflammatory exudation,

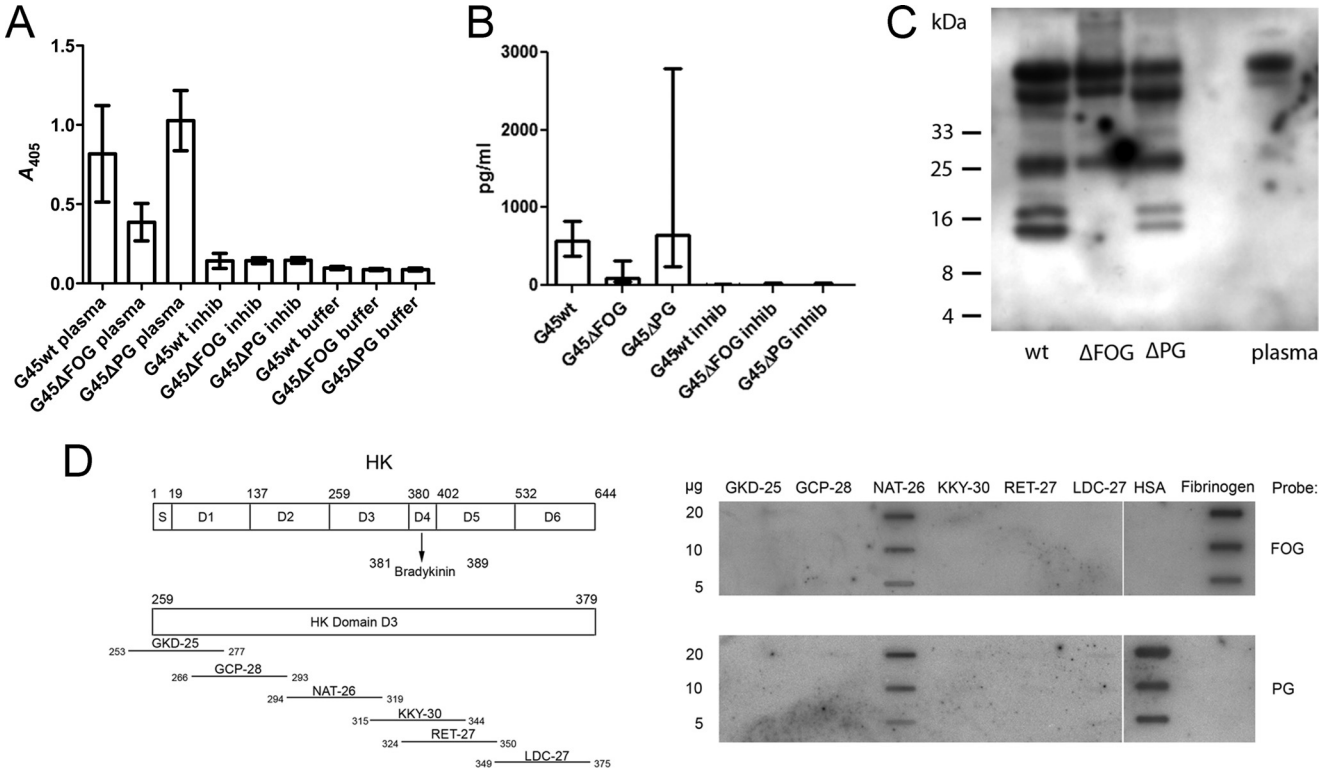

FIGURE 5. **FOG, but not PG, activates the proinflammatory branch of the contact system.** *A*, analysis of contact activation by G45WT, G45ΔFOG, and G45ΔPG in human plasma using a chromogenic assay. Mid-log bacteria were incubated with plasma with or without the synthetic peptide *H*-D-Pro-Phe-Arg-CMK (a specific contact system inhibitor; *inhib*) or with buffer only, washed, and incubated with the chromogenic substrate S-2302. The bacteria were spun down, and A_{405} was determined for the supernatants. Values are the mean ± S.D. for six (samples with or without inhibitor) or four (controls with buffer) experiments. *B*, analysis of BK release by G45WT, G45ΔFOG, and G45ΔPG in human plasma. Mid-log bacteria were incubated with plasma, and the release of BK was measured by ELISA. Median values and the range for five experiments are indicated. The skew for G45ΔPG is due to one extreme outlier. There was a significant difference between strains as assessed by the Kruskal-Wallis test ($H(3) = 8.69$, $p = 0.013$), with G45ΔFOG being different from both G45WT and G45ΔPG (Dunn's multiple comparison test). *C*, mid-log bacteria were incubated with 50% plasma. Adsorbed proteins were eluted from the bacteria with glycine buffer (pH 2.0), followed by TCA precipitation and analysis by Western blotting using antibodies against NAT-26. Low molecular mass NAT-26-containing bands are seen for G45WT and G45ΔPG but not G45ΔFOG bacteria. *D*, binding of ^{125}I-labeled FOG or PG to peptides spanning domain D3 of HK. The overlapping peptides and their positions in domain D3 are depicted below the schematic representation of HK and domain D3. Fibrinogen and HSA were included as positive controls.

the processing of NAT-26-containing HK fragments into smaller peptides in the presence of FOG was confirmed (data not shown). Because NAT-26 has affinity for both FOG and PG, this suggests that smaller antibacterial NAT-26-containing HK fragments are not produced at the surface of G45ΔFOG, *i.e.* PG is insufficient and FOG is required for a more complete activation of the contact system generating these peptides.

It has been reported that antibacterial NAT-26-containing fragments of HK are produced at the surface of GAS (26). The molecular mass of these fragments is in the range of 13–17 kDa, which corresponds well with the peptides identified at the surface of FOG-expressing GGS (Fig. 5*C*). To investigate whether FOG or PG could protect the bacteria against the NAT-26 peptide, G45WT, G45ΔFOG, and G45ΔPG were incubated with NAT-26 at different concentrations. The results demonstrate that the mutant lacking PG was more susceptible to killing by NAT-26 (Fig. 6*A*). Apart from being attached to the bacterial cell wall, FOG and PG are also released into the growth medium (21, 35, 36). Soluble FOG and PG both blocked (with PG more efficiently than FOG) the killing of G45ΔPG bacteria by NAT-26 (Fig. 6*B*). In Fig. 7, electron microscopy was employed to study the morphology of G45WT and the two G45 mutants following incubation with NAT-26 at 2 μM. The micrographs show that among the three isolates, the cell wall architecture of G45ΔPG bacteria was more disintegrated with ejected cytoplasmic material and membrane blebs. Combined, the results demonstrate that PG, in solution or associated with the bacterial cell surface, protects GGS against the killing activity of NAT-26.

In conclusion, previous investigations have demonstrated that several bacterial pathogens activate the contact system, inducing coagulation and inflammation at the site of infection. The consequence(s) of this response for the bacteria is not clear. An inflammatory response will initiate host defense mechanisms such as complement activation and recruitment of phagocytes, and the fact that activation of the contact, complement, and coagulation systems generates antibacterial peptides (26, 37, 38) should also be detrimental. On the other hand, a controlled induction of inflammation and coagulation could be advantageous for bacteria colonizing an epithelial surface. Increased vascular permeability will cause influx of plasma rich in nutrients and could facilitate spread of the infection, whereas the formation of a clot may promote adhesion and provide a protective shield. It is difficult to anticipate which of these contradictory effects prevail at different phases of infection.

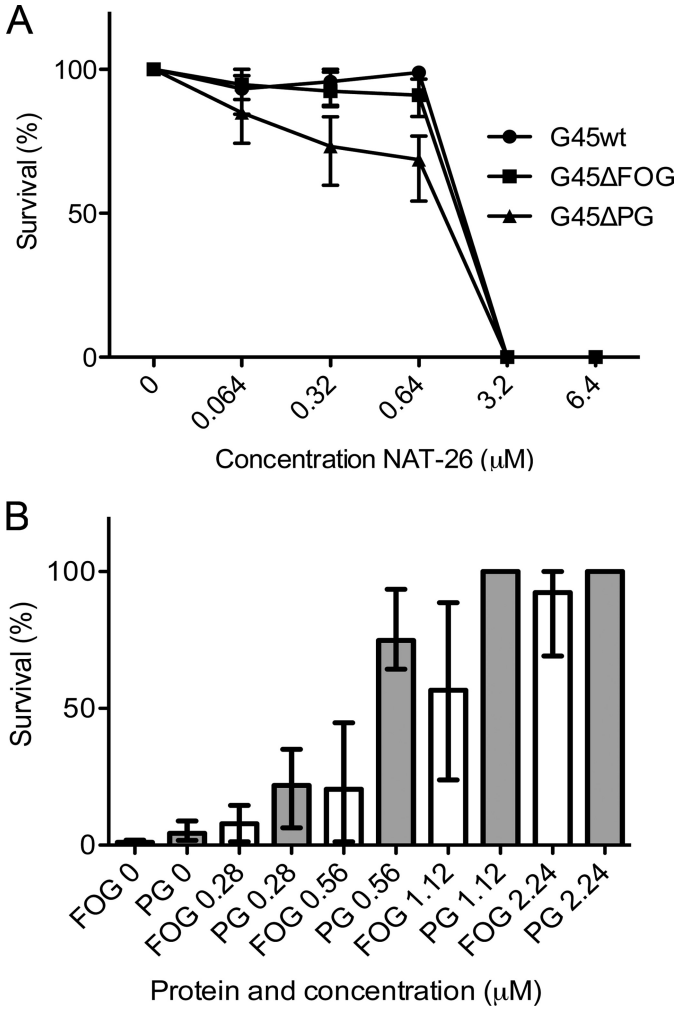

FIGURE 6. **PG protects GGS against antibacterial activity of NAT-26.** *A*, mid-log G45WT, G45ΔFOG, and G45ΔPG bacteria were incubated with various concentrations of NAT-26, and survival was determined by plating. Mean values and the range of three experiments are shown. *B*, the bactericidal effect of NAT-26 at 1.28 μM on G45ΔPG was inhibited with various concentrations of FOG1-C and 35-kDa PG, and survival was determined by plating. Mean values and the range of four (FOG) or three (PG) experiments are shown.

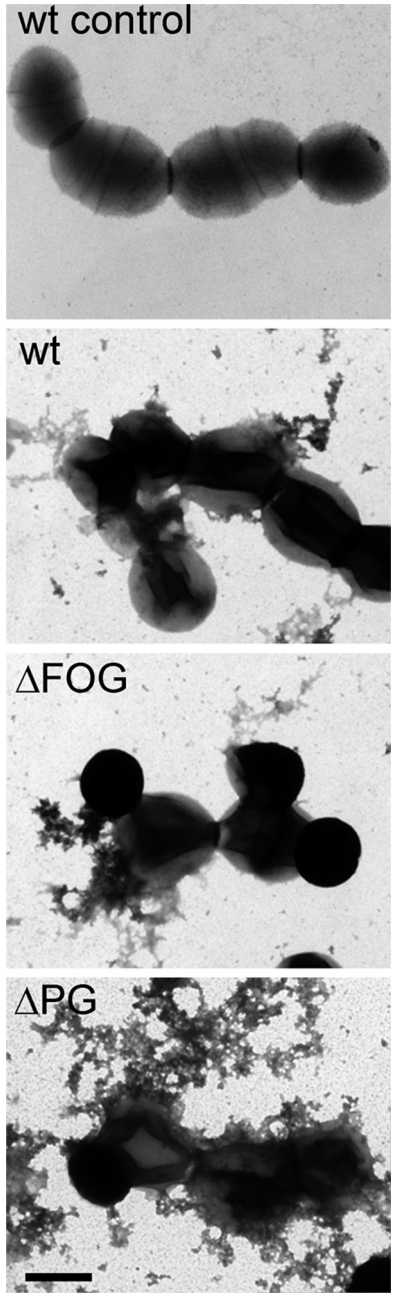

FIGURE 7. **Visualization of antibacterial effect of NAT-26.** Electron micrographs show untreated G45WT and NAT-26-treated G45WT, G45ΔFOG, and G45ΔPG bacteria. *Scale bar* = 1 μm.

The main finding of this study is that FOG and PG activate the contact system but also inhibit (PG) the antibacterial activity of peptides resulting from the activation. A question raised by the present data concerns the degree of inflammation in relation to the severity of streptococcal infection. Although invasive streptococcal infections and rheumatic fever kill hundreds of thousands of individuals annually, with GAS alone at >0.5 million (39), it should be stressed that the vast majority of streptococcal infections are uncomplicated superficial cases of pharyngitis and skin infections and that asymptomatic colonization is even more common. A characteristic feature of severe invasive streptococcal infections is a systemic and massive inflammation, often connected with bleeding disorders, and in relation to the contact system, it is of interest that low levels of FXII and a prolonged aPTT are often seen in patients with septic shock (40). It is unlikely that the rare but clinically highly significant condition with streptococci growing in the bloodstream represents a habitat to which the bacteria have adapted. In comparison, a fine-tuned and well controlled local induction of inflammation at the site of infection appears more adequate, a notion supported by this investigation.

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