

The IL-8 Protease SpyCEP/ScpC of Group A *Streptococcus* Promotes Resistance to Neutrophil Killing

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SUMMARY

Interleukin-8 (IL-8) promotes neutrophil-mediated host defense through its chemoattractant and immunostimulatory activities. The Group A *Streptococcus* (GAS) protease SpyCEP (also called ScpC) cleaves IL-8, and SpyCEP expression is strongly upregulated in vivo in the M1T1 GAS strains associated with life-threatening systemic disease including necrotizing fasciitis. Coupling allelic replacement with heterologous gene expression, we show that SpyCEP is necessary and sufficient for IL-8 degradation. SpyCEP decreased IL-8-dependent neutrophil endothelial transmigration and bacterial killing, the latter by reducing neutrophil extracellular trap formation. The knockout mutant lacking SpyCEP was attenuated for virulence in murine infection models, and SpyCEP expression conferred protection to coinfecting bacteria. We also show that the zoonotic pathogen *Streptococcus iniae* possesses a functional homolog of SpyCEP (CepI) that cleaves IL-8, promotes neutrophil resistance, and contributes to virulence. By inactivating the multifunctional host defense peptide IL-8, the SpyCEP protease impairs neutrophil clearance mechanisms, contributing to the pathogenesis of invasive streptococcal infection.

INTRODUCTION

Group A *Streptococcus* (GAS) is a leading human pathogen responsible for a wide range of diseases ranging from simple pharyngitis and impetigo to life-threatening, invasive conditions such as necrotizing fasciitis (NF) and toxic shock syndrome (TSS). The worldwide burden of invasive GAS infection is estimated at over 650,000 cases and 150,000 deaths annually (Carpatis et al., 2005). While GAS strains of many genotypes are capable of producing invasive infections, strains representing one globally disseminated M1T1 clone have persisted for over 20 years as the most prevalent sterile site isolates (Chatellier

et al., 2000; Cleary et al., 1998; Cockerill et al., 1997; Murono et al., 1999), as corroborated by the nine surveillance centers of the United States Centers for Disease Control Emerging Infections Program Network each year from 1997 through 2005 (<http://www.cdc.gov/ncidod/dbmd/abcs>).

Sumby et al. recently provided a genome-wide analysis using expression microarrays to characterize fundamental differences in the transcriptome of M1T1 GAS isolates isolated from invasive disease cases versus those associated with mucosal site infection (Sumby et al., 2006). This comprehensive analysis showed that a frameshift mutation in the gene *covS*, encoding the sensor kinase component of an important GAS global transcriptional regulator (Dalton et al., 2006; Mitrophanov et al., 2006), resulted in striking effects on GAS gene expression including the upregulation of several proven or candidate virulence factors. A second most highly upregulated gene (25-fold) in the invasive GAS transcriptional profile was that encoding SpyCEP (*S. pyogenes* cell envelope protease). SpyCEP (also known as ScpC and PrtS) is a predicted 1645 amino acid protein that contains a C-terminal LP(X)TG cell wall anchor, and it was recently identified as a protease capable of degrading and functionally inactivating the major human CXC chemokine interleukin-8 (IL-8) (Edwards et al., 2005; Hidalgo-Grass et al., 2006).

IL-8 is a multifunctional host defense protein with a prominent role in recruitment and activation of neutrophils. Not only does IL-8 act as potent chemoattractant (Kunkel et al., 1991), it also tethers itself to the luminal surface of the microvasculature providing a stop signal to rolling neutrophils (DiVietro et al., 2001; Middleton et al., 1997). SpyCEP purified from an M81 GAS NF isolate was found to specifically cleave the IL-8 C terminus between a glutamine residue at position 59 and an arginine residue at position 60, eliminating its chemotactic properties (Edwards et al., 2005); the murine chemokine MIP-2 was cleaved at the same site. Independent studies in a GAS M14 NF isolate (Hidalgo-Grass et al., 2006) revealed that the same protease also efficiently degraded the murine CXC chemokines KC and MIP-2 both in vitro and in vivo, impairing mouse neutrophil recruitment to a greater extent than the related GAS protease ScpA, which specifically targets the human complement-derived chemoattractant C5a (Cleary et al., 1992; Ji et al., 1996).

Due to its marked upregulation in the invasive transcriptional profile, we hypothesized that SpyCEP played an important role

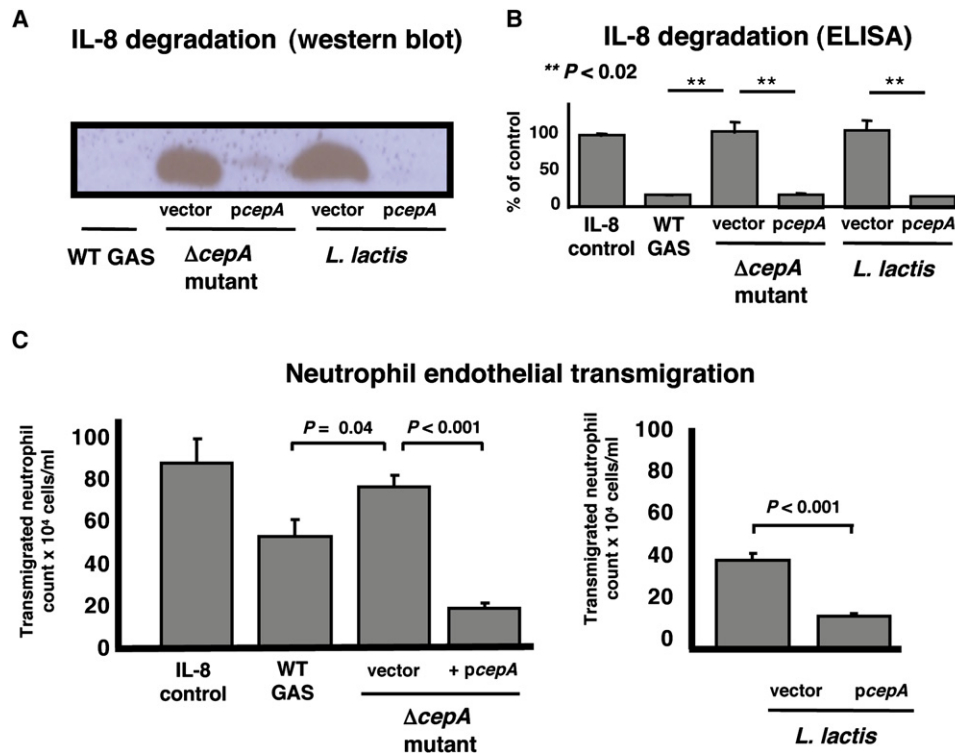


Figure 1. SpyCEP Is Necessary and Sufficient for IL-8 Degradation and Impairment of Neutrophil Recruitment

(A and B) SpyCEP expression was manipulated by targeted mutagenesis of GAS (loss of function), complementation, and heterologous expression in *L. lactis* (gain of function). Presence of SpyCEP correlates with IL-8 protease activity by anti-IL-8 western blot (A) and ELISA (B). Error bars represent mean \pm standard deviation.

(C) SpyCEP degradation of IL-8 impairs human neutrophil transmigration across an endothelial monolayer. Recombinant IL-8 was used as a positive control. Transmigration indicates migration of neutrophils across the endothelium. Transwell experiments were performed in triplicate and repeated three times; pooled data are shown \pm SEM. "Vector" refers to empty plasmid pDCerm to serve as control for complementation analysis.

in M1T1 GAS resistance to phagocytic clearance and animal virulence. To test this hypothesis, we deleted the gene encoding SpyCEP, *cepA*, in an invasive M1T1 NF isolate through precise, in-frame allelic replacement. This loss of function reagent is coupled with heterologous expression of SpyCEP in the non-pathogenic Gram-positive *Lactococcus lactis* to probe potential pathogenic roles of the chemokine protease in promoting GAS resistance to human neutrophil killing, adherence and invasion of epithelial cells, and virulence in murine models of NF and upper respiratory tract infection.

RESULTS

Mutagenesis and Heterologous Expression of SpyCEP

A strategy for precise, in-frame allelic replacement of the *cepA* gene with a chloramphenicol acetyltransferase (*cat*) antibiotic resistance gene cassette was executed successfully in M1T1 GAS strain 5448, an isolate from a patient with NF and TSS, yielding mutant 5448 Δ *cepA* (Figure S1A). The intact M1T1 *cepA* gene was also cloned in the *Escherichia coli*-streptococcal shuttle expression vector pDCerm (Jeng et al., 2003), generating *pcepA*, for use in complementation analysis and heterologous expression of the protease in *Lactococcus lactis*. Western blot analysis detected the presence of SpyCEP in culture supernatants of the WT M1TI GAS parent strain, loss of expression in the isogenic

Δ *cepA* mutant, and overexpression of SpyCEP upon complementation of the mutant or expression in *L. lactis* using the high-copy number *pcepA* plasmid (Figure S1B). SDS-PAGE showed generation of the 6 kD SpyCEP IL-8 cleavage product after coincubation of the supernatants from the WT GAS parent strain, complemented Δ *cepA* mutant, and *L. lactis* expressing *pcepA* with IL-8, but the cleavage product was absent in the Δ *cepA* mutant or *L. lactis* containing the empty vector control (Figure S1C). Western blot analysis (Figure 1A) and IL-8 ELISA (Figure 1B) confirmed IL-8 protease activity of the corresponding strains expressing SpyCEP and its absence in strains lacking the protease. These studies confirm generation of loss-of-function and gain-of-function bacterial reagents with alterations in IL-8 protease activity for analysis of functional interactions with human neutrophils. The Δ *cepA* mutant did not differ from the parent strain in expression of M1 protein (Figure S2A), zone of β -hemolysis surrounding colonies on blood agar plates (not shown), DNase activity (Figure S2B), nor susceptibility to the cationic antimicrobial peptide polymyxin B (MIC = 28 μ g/ml for both).

SpyCEP Impairs Neutrophil Endothelial Transmigration

In response to the chemotactic signal of IL-8, neutrophils will migrate directionally across a human microvascular endothelial cell monolayer, a process we and others have modeled in vitro using a transwell filter insert system (Doran et al., 2003; Edwards

et al., 2005; Yao et al., 1996). Human microvascular endothelial cells (hBMEC) were propagated on transwells until tight junctions were established, living bacteria placed in the bottom well along with a fixed concentration of recombinant IL-8, and freshly isolated human neutrophils added to the upper well. Neutrophil transmigration of the endothelial monolayer toward the GAS $\Delta cepA$ mutant and untransformed *L. lactis* was significantly greater than observed with the WT GAS parent strain, complemented $\Delta cepA$ mutant or *L. lactis* expressing *pcepA* (Figure 1C). These studies confirm that GAS expression of the IL-8 protease can retard directional migration of human neutrophils, and consequently serve to delay the kinetics of the innate immune response.

SpyCEP Promotes Resistance to Neutrophil Killing

The very strong upregulation of *cepA* expression in the invasive M1T1 GAS transcriptome suggested potential benefits to bacterial survival beyond interference with neutrophil recruitment. IL-8, formerly known as neutrophil activating peptide-1, is produced in large amounts by neutrophils themselves upon encountering bacteria (Bazzoni et al., 1991; Strieter et al., 1992), and in an autocrine fashion can exert stimulatory effects on oxidative burst function and release of granule enzymes amplifying bactericidal activity (Simms and D'Amico, 1997; Thelen et al., 1988). We assessed the contribution of SpyCEP to bacterial survival in a human neutrophil opsonophagocytic killing assay. WT parent M1T1 GAS survived neutrophil killing 6-fold more than $\Delta cepA$ mutant bacteria ($p < 0.005$); WT levels of neutrophil resistance were restored by complementation of the mutant with the *pcepA* expression vector (Figure 2A, left). Heterologous expression of SpyCEP in *L. lactis* increased survival of the transformed bacterium in the neutrophil killing assay by ~50% ($p < 0.005$) (Figure 2A, right). Consistent with prior results (Hidalgo-Grass et al., 2006), SpyCEP did not promote survival in human whole blood (Figure S3A), suggesting its action to be predominantly exerted at localized tissue sites of infection.

SpyCEP Reduces Neutrophil Production of Extracellular Traps

Apart from their phagocytic function, neutrophils can efficiently capture and kill microbes in the extracellular space by extrusion of a matrix of DNA and histones known as neutrophil extracellular traps, or NETs, which ensnare bacteria and subject them to microbicidal effectors including elastase and myeloperoxidase (Brinkmann et al., 2004). M1T1 GAS express DNases including the phage-encoded Sda1 that contribute to NET degradation and immune evasion (Buchanan et al., 2006; Sumby et al., 2005; Walker et al., 2007). NETs are induced by exposing human neutrophils to IL-8 (Gupta et al., 2005). We thus hypothesized that the IL-8 degradation by SpyCEP could reduce NET formation and thus bacterial killing, suggesting another mechanism by which the IL-8 protease could impair host defense function. Using a fluorescent assay for real-time visualization of NETs (Buchanan et al., 2006), we confirmed the capacity of the chemokine to stimulate NET formation by human neutrophils (Figure 2B). We then visualized NET formation elicited by our isogenic bacterial strains in the presence of the inhibitor G-actin to block DNase-mediated NET degradation. As shown in Figure 2B, NETs were significantly more abundant in human neutrophils incubated with the $\Delta cepA$ mutant than with the isogenic WT parent

strain or the complemented mutant; conversely, NET production was diminished in neutrophils exposed to *L. lactis* expressing SpyCEP compared to *L. lactis* transformed with empty vector control. A significant contribution of SpyCEP to M1 GAS survival was maintained when phagocytosis was inhibited with cytochalasin D, confirming a role in the pathogen's resistance to extracellular neutrophil killing mechanisms (Figure S3B). Furthermore, a significant contribution of SpyCEP to GAS neutrophil survival was also maintained when NET degradation was inhibited with G-actin (Figure S3B). This indicates SpyCEP action affects NET production rather than NET degradation, consistent with the observation that WT and $\Delta cepA$ mutant GAS exhibit similar DNase activity (Figure S2B). Also consistent with the hypothesis that SpyCEP blocks NET production by ablating IL-8-induced NET formation, addition of excess exogenous IL-8 to the assay eliminated the differences in NET induction between WT and $\Delta cepA$ mutant strains (Figure S3C).

SpyCEP Contributes to GAS Virulence in a Murine Necrotic Skin Infection Model

A murine subcutaneous challenge model can be used to examine the contribution of specific bacterial phenotypes to the virulence of M1T1 GAS in vivo (Buchanan et al., 2006; Datta et al., 2005; Sumby et al., 2005). Using inbred C57Bl6 mice, we injected one shaved flank with 5×10^7 cfu of the WT M1T1 GAS parent strain and the contralateral flank with an equivalent inoculum of the isogenic $\Delta cepA$ mutant. As shown in the graph and representative photo of Figure 3A, the mean lesion size generated by the $\Delta cepA$ mutant was ~50% that of the WT lesions ($p < 0.02$). Histopathologic analysis showed increased neutrophil recruitment in lesions generated by the $\Delta cepA$ mutant compared to the WT parent strain (Figure 3B), confirming earlier indications that SpyCEP can impair murine neutrophil recruitment in vivo due to its shared activity against murine C-X-C chemokines KC and MIP-2 (Hidalgo-Grass et al., 2006).

We next asked whether SpyCEP protection against neutrophil killing was only afforded to individual bacterial cells themselves expressing the surface-anchored IL-8 protease (action in *cis*), or whether SpyCEP-mediated chemokine degradation could protect other vulnerable bacteria in the local environment (action in *trans*). To address this question, we performed a coinfection experiments in which an equal concentration of WT M1 GAS and isogenic $\Delta cepA$ mutant were inoculated in the same lesion site. When lesions were biopsied and harvested 4 days later, no survival advantage of the WT strain versus the mutant was detected (Figure 3C), indicating the WT strain could rescue the virulence defect of the mutant bacteria, presumably by decreasing neutrophil recruitment, phagocytic ability, and NET formation at the local site of infection. To extend this line of investigation, we adopted a model of polymicrobial necrotizing infection, using a vancomycin-resistant isolate of the nosocomial pathogen *Enterococcus faecalis* (VRE), a frequent isolate from mixed etiology wound and necrotizing soft tissue infections including Fournier's gangrene (Kuo et al., 2007; Ou et al., 1993). Necrotic skin lesions produced by the WT M1 GAS + VRE were significantly larger than those produced by the GAS $\Delta cepA$ mutant + VRE, and it was shown that the presence of the WT strain (with SpyCEP expression) promoted survival of VRE (in vivo and in *trans*) in the mixed infection model (Figure 3D).

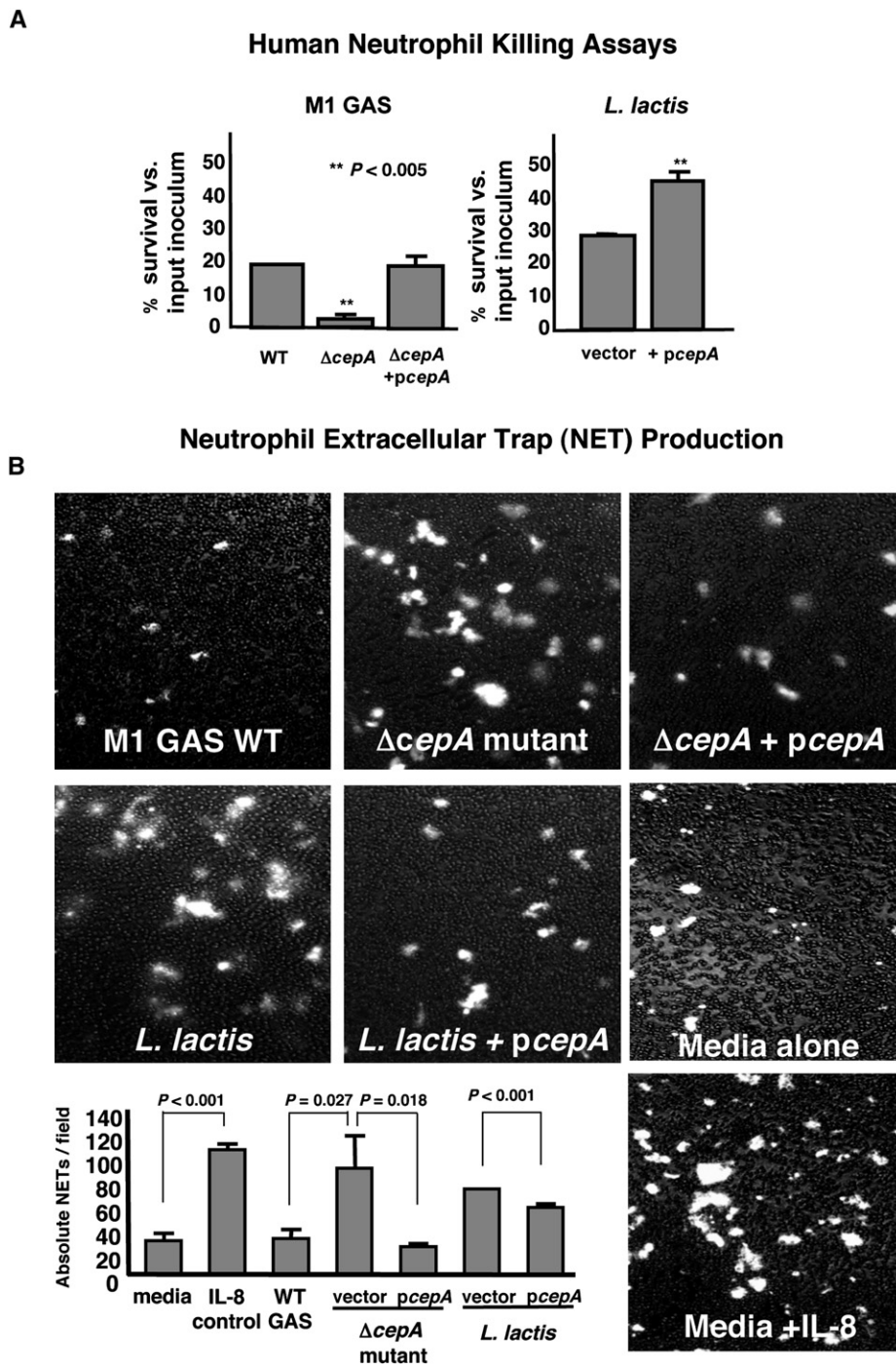


Figure 2. SpyCEP Promotes GAS Resistance to Neutrophil Killing

(A) SpyCEP contributes to GAS survival and increases *Lactococcus lactis* survival upon coincubation with human neutrophils, experiments performed in triplicate and repeated three times with similar results; representative experiment \pm SEM.

(B) The presence of SpyCEP reduces IL-8-mediated induction of neutrophil extracellular traps (NETs) in response to bacterial exposure. Shown are fluorescent images of NETs stained with Sytox Orange and overlaid upon brightfield image of neutrophils. Also shown are quantitative enumeration of NETs; experiments performed in triplicate and repeated three times with similar results; representative experiment \pm SEM, two-sided t test.

SpyCEP Contributes to GAS Virulence following Intranasal Infection

As GAS is also an important cause of upper and lower respiratory tract infections, we next assessed the contribution of SpyCEP to

virulence following intranasal inoculation. We observed the WT M1 strain to have a marked advantage in establishing upper respiratory tract infection compared to the $\Delta cepA$ mutant (Figure 3E). In contrast to subcutaneous infection, this challenge

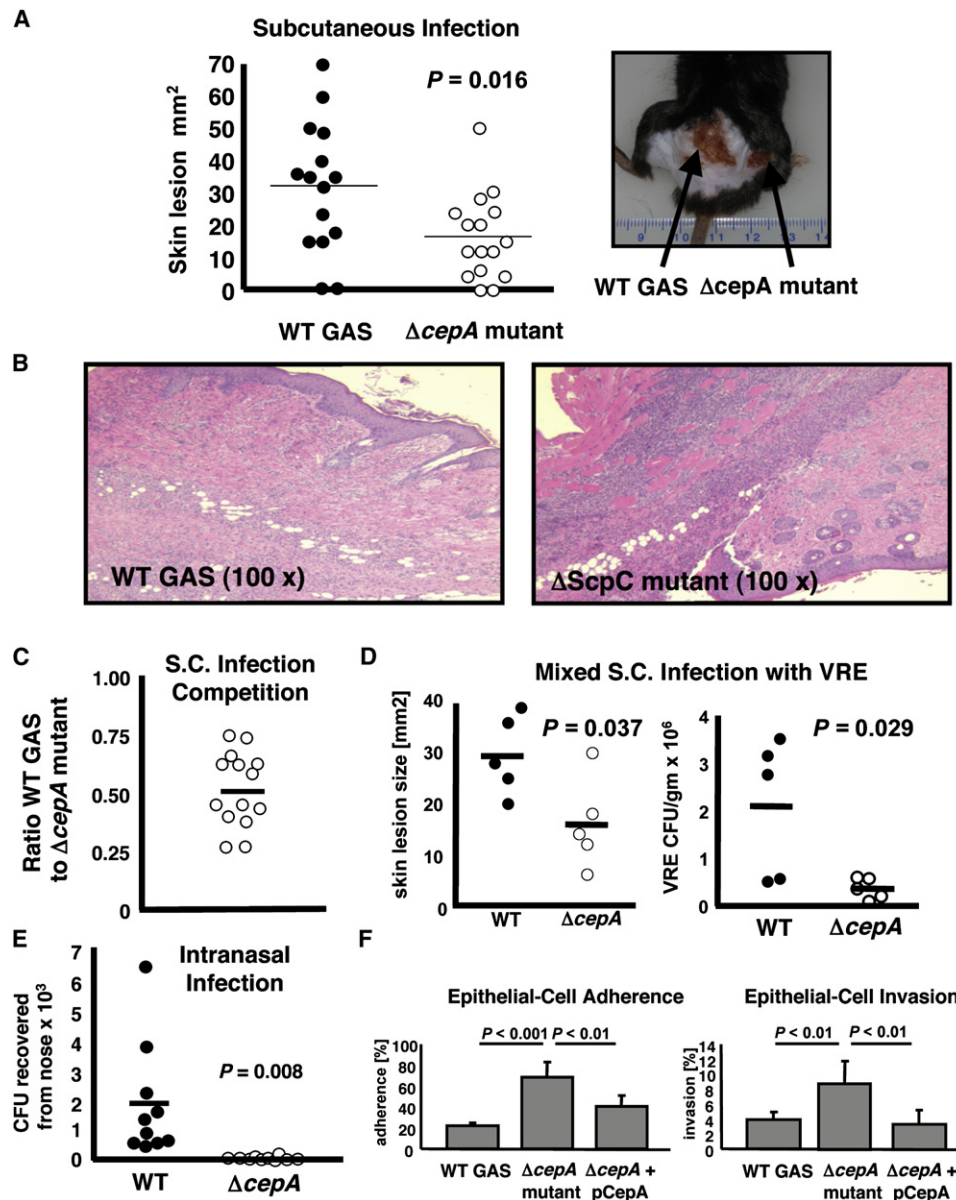


Figure 3. SpyCEP Contributes to GAS Virulence in a Murine Infection Models

(A) C57B16 mice were injected subcutaneously with equivalent inocula of the WT M1 GAS strain (left flank) and the isogenic $\Delta cepA$ mutant (right flank) and skin lesion progression measured for 4 days; a representative photograph of skin lesions observed is shown.

(B) Representative histology (H&E stain) biopsies from skin lesions showing increased neutrophil recruitment at the $\Delta cepA$ mutant infection site (100x magnification).

(C) In a competition experiment, WT M1 GAS and isogenic $\Delta cepA$ mutant were injected simultaneously s.c. injection and bacteria enumerated after 4 days; ratio WT M1 GAS to isogenic $\Delta cepA$ mutant is shown.

(D) GAS SpyCEP expression supports survival in vivo of vancomycin resistance *Enterococcus faecalis* (VRE) in a mixed infection model.

(E) SpyCEP contributes to GAS mucosal infection in an intranasal inoculation model.

(F) SpyCEP reduces GAS epithelial cell adherence and invasion. Elimination of SpyCEP increases the adherence and invasion of HEp-2 human pharyngeal epithelial cells by M1 GAS. Experiments were performed in triplicate and repeated three times with similar results; representative experiment is shown \pm standard deviation.

model involves interaction of GAS with epithelial cells. This raises the possibility that in addition to immune resistance, changes in epithelial cell binding of the $\Delta cepA$ mutant compared to WT GAS could contribute to the observed in vivo attenuation, especially since *cepA* shares ~30% sequence identity and ~50% se-

quence similarity with the GAS C5a peptidase, *ScpA*, which may function as a GAS epithelial cell invasin (Purushothaman et al., 2004). We therefore examined the contribution of SpyCEP to GAS adherence to and invasion of cultured HEp-2 human pharyngeal epithelial cells. The isogenic $\Delta cepA$ mutant was found to

be ~3-fold more adherent ($p < 0.001$) and ~2-fold more invasive ($p < 0.01$) than the GAS WT parent strain; complementation of the mutant with the *pcepA* expression plasmid reversed the adherence and invasion phenotype to nearly WT levels (Figure 3F). These results show that in contrast to *ScpA*, SpyCEP does not act as a GAS epithelial cell adhesin or invasin. An underlying mechanism may be found in the recent observation that GAS is capable of triggering IL-8 release from Hep-2 cells, and that the cytokine can promote GAS entry into cells (Nobbs et al., 2007); SpyCEP degradation of IL-8 would interfere with this pathway. In sum, we conclude that chemokine and neutrophil mediated host defense are operating in the nasal infection model, and whatever contribution SpyCEP contributes to reduce GAS epithelial adherence is outweighed by its ability to blunt immune clearance in vivo.

The Zoonotic Pathogen *Streptococcus iniae* Possesses a Functional SpyCEP Homolog

The β -hemolytic species *Streptococcus iniae* is an important pathogen in wild and aquacultured fish, where it can produce a fatal meningoencephalitis (Agnew and Barnes, 2007). *S. iniae* is also recognized as an emerging zoonotic pathogen of humans, producing invasive cellulitis in fish handlers (Weinstein et al., 1997). By whole genome pyrosequencing and bioinformatic analysis, we identified an *S. iniae* gene encoding a 1631 amino acid (aa) candidate protein sharing 54.4% aa identity and 66.2% aa similarity to SpyCEP of GAS, which we named *CepI* (cell envelope proteinase of *S. iniae*) (Figure S4). The genomic position of *cepI* is consistent with *cepA* in GAS—downstream from a lactate oxidase gene, *LctO* (previously characterized in *S. iniae*) (Gibello et al., 1999), and upstream of genes with high similarity to *spy_0421* and *metG* of M1 GAS (Ferretti et al., 2001). Like GAS, *S. iniae* is virulent in the murine subcutaneous infection model (Fuller et al., 2001). Targeted plasmid integrational mutagenesis of the *cepI* gene in virulent *S. iniae* strain K288 was performed (Figure S5A). Compared to the WT parent strain, the *S. iniae* $\Delta cepI$ mutant lost the ability to degrade IL-8 (Figure S5B), showed increased sensitivity to human neutrophil killing (Figure S5C), and generated smaller lesions in the murine subcutaneous infection model (Figure S5D). Thus, a functional homolog of SpyCEP is present in at least one additional bacterial species, where it contributes as it does for GAS to chemokine degradation, neutrophil resistance and disease pathogenesis.

DISCUSSION

The prominence of GAS among bacteria able to cause serious systemic infections even in previously healthy humans reflects the diverse capacities of this pathogen to avoid eradication by phagocyte defenses of the innate immune system (Kwinn and Nizet, 2007; Voyich et al., 2004). Resistance to neutrophil killing involves the antiphagocytic properties of classical GAS virulence factors such as the surface M protein and hyaluronic acid capsule and cytolytic action of the pore-forming toxins streptolysin S and streptolysin O. In the globally disseminated GAS M1T1 clone, accumulation of a larger repertoire of virulence factors such as the streptococcal inhibitor of complement (SIC) protein and the phage-encoded DNase Sda1 provide added capabilities to resist effective opsonization and escape extracellular killing in

NETs, finding corroboration in epidemiologic associations to severe disease including NF.

In the present study, we establish molecular Koch's postulates for the contribution of IL-8 protease SpyCEP to impaired neutrophil recruitment and resistance to neutrophil killing, including single gene complementation analysis and heterologous expression studies. M1T1 expression of SpyCEP had the anticipated effect of delaying neutrophil endothelial transmigration and recruitment to the tissues and promoting virulence (Edwards et al., 2005; Hidalgo-Grass et al., 2006; Sumby et al., 2008). These results find clinical correlation in the striking paucity of neutrophils at the site of infection that was observed in a subset of human cases of GAS NF consistent with a primary deficit in chemoattractant function (Cockerill et al., 1998; Hidalgo-Grass et al., 2004). Likewise, in a baboon model of GAS soft-tissue infection, the risk of septicemia and mortality was inversely correlated to the magnitude of neutrophil influx at the site of infection (Taylor et al., 1999). However, in advanced human necrotic disease, prominent neutrophil infiltrates ultimately accumulate in association with proliferating bacteria (Dahl et al., 2002), as could be expected since additional microbial components (e.g., fMLP) and host inflammatory response proteins (e.g., C5a, platelet activating factor) possess strong neutrophil chemoattractant properties (Wagner and Roth, 2000).

Our data suggest the proteolytic inactivation of IL-8 by SpyCEP can exert additional effects to impair neutrophil killing of M1T1 GAS even in late stage necrotic lesions. By eliminating IL-8-induced NET production, SpyCEP can synergize with the M1T1 DNase Sda1 (Buchanan et al., 2006; Sumby et al., 2005) to impair NET-mediated extracellular bacterial killing. The cumulative effect of these activities is consistent with our observation that GAS expression of SpyCEP was necessary and sufficient to promote bacterial survival in killing assays with isolated neutrophils, and for GAS to protect non-SpyCEP producing bacteria from neutrophil killing in vivo (Figure 2). The specificity of our assays was aided by the achievement of single gene replacement of *cepA* (independent of *scpA*) and single gene complementation that were technically unsuccessful, respectively, in other recent informative studies of the GAS IL-8 protease activity (Hidalgo-Grass et al., 2006; Sumby et al., 2008). Nevertheless, it is likely that bacterial and mouse strain specificities influence the observed patterns of disease progression, since inflammatory lesion size may also reflect a contribution from host neutrophils that could be augmented in the absence of SpyCEP chemokine degradation (Sumby et al., 2008).

The gene encoding SpyCEP was one of the most highly differentially expressed upon comparison of the transcriptional profile of invasive M1T1 GAS isolates versus similar clonal strains isolated from the pharyngeal mucosa (Sumby et al., 2006). In simple colonization, our results indicate SpyCEP may decrease epithelial cell adherence and, therefore, its expression would not be favored. The observed 25-fold upregulation of *cepA* expression in the invasive transcriptional profile of M1T1 GAS strains is consistent with our findings linking SpyCEP expression to resistance to neutrophil killing, a selective pressure induced uniquely in vivo. Our discovery and analysis of a functional SpyCEP homolog in *S. iniae* may likewise explain the human disease potential of this zoonotic pathogen. Strategies directed at neutralizing SpyCEP activity could prove useful as an adjunctive therapy to

invasive GAS infection, acting not to kill the bacterium directly, but rather to render it susceptible to our natural innate defenses.

EXPERIMENTAL PROCEDURES

Bacterial Strains

WT GAS M1T1 strain 5448 was originally isolated from a patient with NF and TSS (Chatellier et al., 2000). *S. iniae* strain K288 was isolated from the brain of a moribund hybrid striped bass at the Kent SeaTech aquaculture facility (Buchanan et al., 2005). Also used were vancomycin-resistant *Enterococcus faecalis* (VRE) (ATCC 51299) and nonpathogenic *L. lactis* strain NZ9000. GAS, *S. iniae*, *L. lactis*, and VRE were grown in Todd-Hewitt broth (THB) (Difco, BD Diagnostics) or TH agar plates, using 1 µg/ml chloramphenicol (Cm) or 2 µg/ml erythromycin (Em) selection where appropriate. *E. coli* TOP10 (Invitrogen) were grown in Luria-Bertani (LB) broth or LB agar plates using 100 µg/ml ampicillin, 500 µg/ml Em, or 5 µg/ml Cm selection. GAS and *L. lactis* were made competent for transformation by electroporation by growth in THB + 0.6% glycine. For use in neutrophils, whole blood, epithelial cell, and mouse challenge studies, bacteria were grown to logarithmic phase in THB (OD₆₀₀ = 0.4 = ~2 × 10⁸ cfu/ml), pelleted, washed, and resuspended in PBS or tissue culture media at the desired concentration.

Allelic Exchange Mutagenesis of GAS

PCR was used to amplify upstream and downstream DNA fragments from the M1 GAS 5448 chromosome immediately flanking the *cepA* gene. Primers pairs used were (1) *cepA*-upF (5'-ggataagacgggtcaaca-3', 526 bp upstream of the *cepA* start codon) with *cepA*-upR (5'-gggtgatataccagtgatttttccactctga taccctcctaattgtt-3'; immediately upstream of the *cepA* start site + 30 bp extension corresponding to 5' end of the *cat* gene) and (2) *cepA*-downF (5'-tactgcgatgagtgccagggcgggcgtaataacaagcgcaagagaca-3'; immediately downstream of the *cepA* stop codon + 30 bp extension corresponding to the 3' end of the *cat* gene) with *cepA*-downR (5'-gtcacatcagcagagctgtt-3'; 526 bp downstream of the *cepA* stop codon). PCR was performed using the two amplicons and an amplicon of the *cat* gene to yield a fusion product in which *cat* replaced *cepA* precisely in GAS chromosomal context. This PCR product was TA cloned into the Gateway entry vector, pCR8/GW/TOPO (Invitrogen), then subcloned into pKODestErm, a derivative of pHY304 constructed to allow L-R combination of gene cassettes, thus generating knockout vector pKO-*cepA*. This plasmid was transformed into GAS M1 and single recombination events identified at 37°C under Em + Cm selection. Selection was relaxed by serial passage at 30°C without antibiotics and double-crossover events identified by screening for colonies with Cm^R but Em^S phenotype (Figure S1A). Precise, inframe allelic exchange of *cepA* with *cat* in the GAS 5448Δ*cepA* mutant was confirmed by PCR analysis. M protein dot blot analysis was performed on serial 10-fold dilutions of GAS cells using antibodies directed at the N-terminal domain of the M1 protein (Chatellier et al., 2000). Polymyxin B MIC was determined on logarithmic phase bacteria after 24 hr incubation with the peptide at 37°C. DNase activity was tested by coincubating the supernatants of logarithmic phase bacteria (OD 0.4) with calf thymus DNA (1 µg/µl) and buffer (300 mM Tris, 3 mM CaCl₂, 3mM MgCl₂) for 15 min. The reaction was stopped by adding 0.33 M EDTA and the extent of DNA degradation visualized by electrophoresis on a 1% agarose gel.

Complementation Studies and Heterologous Expression of *cepA*

The M1 GAS *cepA* gene and flanking was amplified by PCR from the GAS chromosome using primers *cepA*-upF and *cepA*-downR, TA cloned into the Gateway-TOPO vector, then subcloned using L-R recombinase into pDestErm, a version of pDCerm (Jeng et al., 2003) engineered to contain L-R recombination sequences in its multiple cloning site, generating expression vector p*cepA*. This plasmid was electroporated into the GAS 5448Δ*cepA* mutant and *L. lactis* strain NZ900 and transformants identified by Em^R phenotype, and confirmed by PCR and restriction analysis. Plasmid pDestErm was introduced into the corresponding strains as an empty vector control.

Mutagenesis of *S. iniae* Ceph

Whole genome pyrosequencing (454 Life Sciences) of the virulent *S. iniae* isolate K288 generated short sequence contigs with high BLAST similarity to the

GAS *cepA* gene (Hidalgo-Grass et al., 2006). Using preliminary public data from the Baylor College of Medicine Human Genome Sequencing Center *S. iniae* genomic sequencing project (<http://www.hgsc.bcm.tmc.edu>), we identified the full putative *S. iniae* *ceph* gene and designed primers for mutagenesis. The middle third of *S. iniae* *ceph* was amplified by PCR using primers cep1-1632F (5'-aggctgtttacgattgaaag-3') + cep1-3284R (5'-tcttcaaccatataataagaag-3'). The 1653 bp amplicon was cloned into pCR8GW/TOPO and then subcloned via LR recombination into pKODestErm, as described for generation of the GAS pKO-*ceph* knockout plasmid. Electrocompetent WT *S. iniae* K288 were transformed with the pKO-*ceph* plasmid. Positive Em^R transformant colonies growing at 30°C were temperature shifted to 37°C to select for bacteria that underwent a single-crossover event in which the pKO-*ceph* was linearized into the chromosome, disrupting the native *ceph* gene (Figure S5A). Insertion mutants were confirmed through PCR analysis. For comparative analyses with the Em^R *ceph* mutant, Em^R was introduced into WT K288 through transformation with the empty pDestErm complementation vector (Locke et al., 2007). For functional assays, *S. iniae* strains were grown at 30°C on THA agar or in THB liquid media containing 5 µg/ml Em.

Western Blot and ELISA Analysis

For IL-8 detection, bacteria were grown to midlogarithmic phase, washed with PBS, and resuspended in RPMI + 0.1% fetal calf serum (FCS). Human IL-8 (R&D Systems) was added to the bacterial suspensions at final concentration of 1 mg/ml. After 4 hr (GAS) and 24 hr (*S. iniae*) incubation at 37°C, the samples were loaded and separated on a 4%–12% Tris-tricine in MES buffer (Invitrogen) and transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk + 0.2% Tween-20. Detection used primary antibody mouse anti-human monoclonal IL-8 (R&D Systems), secondary antibody peroxidase-conjugated sheep anti-mouse (GE Healthcare UK Limited), and detected using the Supersignal West Pico Chemiluminescent System (Pierce). For detection of SpyCEP protein by GAS and *L. lactis* strains, rabbit polyclonal antibody raised against a recombinant protein representing residues 35–587 of the pre-pro SpyCEP enzyme sequence at 1:1000 dilution was employed and developed using Protein G-peroxidase (Sigma) and the ECL system. To monitor IL-8 cleavage, 10 µl of cell-free supernatant from an overnight culture of each bacterial strain was coincubated with an equal volume of human IL-8 (final concentration 5 µg/mL) at 37°C. Samples were then electrophoresed on a 12% Bis-Tris SDS PAGE gel (Invitrogen) in MES buffer and stained using the colloidal blue staining kit (Invitrogen). Quantitative ELISA for IL-8 was performed using the Quantikine kit (R&D Systems) as described previously (Hidalgo-Grass et al., 2004).

Endothelial Transcytosis Assay

Transmigration of human neutrophils across polar human brain microvascular endothelial cell (hBMEC) monolayers was assessed using an adaptation of a published assay (Chen et al., 2006). Briefly, polar hBMEC monolayers were established on collagen-coated TranswellTM plates (6.5 mm diameter, 3 mm pore size, Biocoat, BD Biosciences). Human neutrophils were purified from healthy donors using the PolymorphPrepTM system (Axis-Shield, Fresenius) and 1 × 10⁶ cells added to the upper well of the chamber. Midlog phase GAS or *L. lactis* at 1 × 10⁷ cfu/ml were pre-incubated with 70 ng/ml of recombinant human IL-8 for 2 hr at 37°C, then added to the lower well in 600 µl volume. Recombinant human IL-8 (40 ng) was added to the lower well as a positive control. Plates were incubated for 150 min at 37°C in 5% CO₂ before direct enumeration of neutrophils transcytosing to the lower well in a counter chamber (average of 4 high-power fields).

Neutrophil and Whole-Blood Killing Assays

Neutrophils were isolated from the blood of healthy volunteers as above. Logarithmic phase bacteria were preopsonized in 80% autologous human plasma for 30 min at 37°C, added to neutrophils at final multiplicity of infection (MOI) = 2.5 bacteria/neutrophil, then incubated for 30 min at 37°C with orbital rotation. In stated assays, G-actin 100 µg/ml, to inhibit DNase activity, or cytochalasin D 10 µg/ml, to inhibit phagocytosis, were added to the neutrophils during 10 min preincubation before addition of bacteria. Neutrophils were then lysed with sterile H₂O and serial dilutions plated on THA for enumeration of surviving bacterial cfu. For visualization of NETs, bacteria were mixed with neutrophils at MOI = 0.1 in the presence of G-actin 100 µg/ml for 5 min to inhibit GAS DNase

activity (Buchanan et al., 2006), 0.1 mM Sytox orange stain (Molecular Probes, Invitrogen) for extracellular DNA added, and the mixture immediately visualized without fixation or washes using a Nikon TE200 inverted fluorescent microscope with image capture by CCD camera. In stated assays exogenous IL-8 was added at a final concentration of 2.5 $\mu\text{g}/\mu\text{L}$. For quantification NETs were enumerated by counting three transects of three independent wells after staining with Sytox orange. For whole-blood killing, phlebotomy was performed on healthy volunteers, and 10^4 cfu of midlog phase GAS in 100 μl were added to 300 μl whole blood. Aliquots were plated on THB agar after 15 and 30 min for enumeration of surviving GAS cfu.

Epithelial Cell Adherence and Invasion Assays

HEp-2 human pharyngeal epithelial cells were cultured in RPMI media + 10% FCS, split into 24-well plates, and allowed to grow to confluence for 48 hr prior to assays. Using MOI = 5 bacteria/cell, cellular adherence and antibiotic protection (invasion) assays were performed as described (Timmer et al., 2006). All assays were performed in quintuplicate and repeated three times with similar results. For live cell imaging, HEp-2 cells were incubated with bacteria as described for the adherence assay; however, the bacteria were prestained for 30 min using Fluorescein isothiocyanate Isomer I (Fluka, Sigma-Aldrich). At the end of the assay, wells were treated for 10 min with the fish antimicrobial peptide moronecinin (Lauth et al., 2002) at final concentration 4 μM to kill all extracellular bacteria and Sytox Orange added to final concentration 0.1 μM for staining of dead cells. Cells were visualized using a Nikon TE200 inverted microscope with appropriate fluorescent filters and images captured with a CCD camera.

Mouse Infection Models

GAS and *S. iniae* virulence was tested using an established murine model of necrotizing skin infection (Buchanan et al., 2006; Nizet et al., 2001). Briefly, logarithmic phase GAS or *S. iniae* were resuspended in PBS, mixed 1:1 with sterile Cytodex beads (Sigma, St. Louis, MO). An inoculum of 5×10^7 cfu of WT GAS or *S. iniae* was injected subcutaneously into one flank of 10- to 12-week-old C57Bl6 mice (Charles River Laboratories, CA), and simultaneously an equivalent inoculum of the isogenic ΔcepA (ΔcepI) mutant was injected into the contralateral flank, allowing each mouse (N = 16, GAS; N = 6, *S. iniae*) to serve as its own control. The size of developing necrotic lesions was monitored daily for 4 days, at which time mice were euthanized and histopathology performed on formalin-fixed biopsy samples. Differences in lesion size were analyzed using the paired Student's t test. For a co-infection competition model, 5×10^7 cfu of a 50:50 mix of WT GAS and isogenic ΔcepA GAS mutant was injected subcutaneously into the shaved flank of C57Bl6 mice (N = 14); on day 4, the skin was collected in order to enumerate the surviving bacteria with strain determination by antibiotic selection and PCR confirmation. For the polymicrobial infection model, 2.5×10^7 cfu of WT GAS or the isogenic ΔcepA GAS mutant were mixed with an equivalent number of VRE and injected subcutaneously into the shaved flank of C57Bl6 mice (N = 5); on day 4 the skin was collected in order to enumerate the surviving bacteria. For upper respiratory tract mucosal infection, 10- to 12-week-old C57Bl6 mice (N = 10 per group) were infected intranasally with 1×10^8 cfu of WT GAS or isogenic ΔcepA GAS mutant, respectively. After 4 days, the mice were euthanized, nose excised and homogenized, and the surviving intranasal bacteria enumerated. Differences were analyzed using an unpaired Student's t test.

SUPPLEMENTAL DATA

Supplemental Data include five figures and Supplemental References and can be found online at <http://www.cellhostandmicrobe.com/cgi/content/full/4/2/170/DC1/>.

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