UNIVERSITY OF CALIFORNIA, SAN DIEGO

Virulence Mechanisms of Group A *Streptococcus*

A dissertation submitted in partial satisfaction of the
requirements for the degree of Doctor of Philosophy

in

Molecular Pathology

by

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The Dissertation of Anjuli M. Timmer is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

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DEDICATION

To my family

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LIST OF COMMON ABBREVIATIONS

BMDM	bone marrow-derived macrophage
cfu	colony forming units
Cm	chloramphenicol
DNase	deoxyribonuclease
ELISA	enzyme-linked immunosorbant assay
Em	erythromycin
FBS	fetal bovine serum
GAS	Group A *Streptococcus*
hBMEC	human brain microvascular endothelial cell line
HEp-2	human pharyngeal epithelial cell line
i.p.	intraperitoneal
i.v.	intravenous
IL-1β	interleukin 1 beta
IL-8	interleukin 8
J774	murine macrophage cell line
KCl	potassium chloride
L. lactis, LL	*Lactococcus lactis*
mg	milligram
μg	microgram
Ml	milliliter
μl	microliter
MOI	multiplicity of infection
NADase	nicotinamide dehydrogenase
NETs	neutrophil extracellular traps
NF	necrotizing fasciitis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMN	polymorphonuclear cells
SfbX	Streptococcal fibronectin binding protein X
SLO	streptolysin O
SOF	serum opacity factor
SpyCEP	*Streptococcus pyogenes* cell envelope protein
THA	Todd-Hewitt agar
THB	Todd-Hewitt broth
TMRE	tetramethylrhodamine ethyl ester
TNFα	tumor necrosis factor alpha
TSS	toxic shock syndrome
TUNEL	terminal dUTP nick-end labeling
WT	wild-type
$\Delta\psi_M$	mitochondrial membrane potential

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As time has passed in the Nizet lab I have become increasingly aware of how lucky I am. Listening to stories from other graduate students and postdocs of their experiences has opened my eyes to how fortunate we are here. Victor Nizet is an outstanding mentor and friend. My time in this lab has been filled with encouragement and support, motivating me to work hard and have a passion for my research. Victor has provided me with excellent opportunities for collaborating, finding funding, learning new skills, and thinking independently while still under his guidance. During graduate school I have participated in student government, gotten married, and had a son – all with the support and understanding of Victor. I was lucky to have a P.I. who cares so much about the people in his lab – what they want for their careers and what they want for their lives. I have learned a great deal about being a caring and effective leader from Victor and I hope that I can someday have the same effect on others in my life.

Of course, I am not the only one who knows how great this lab is, and so many wonderful people have flocked here and have made my time here unforgettable. I have seen the lab grow exponentially and every person who has come through has had an impact on my thesis. I will never forget the lab debates about cakes vs. pies, political discussions, Hare Krishna lunches, Porter's Pub happy hours, CuriosiTea, broomball, baseball games, pranks, buffet lunches, journal clubs, parties, conferences, and the fun times we had just being in the lab working together. There are a few people early on that had a major impact on my

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he might be trying to thwart me, but just having his smiles and his love is enough to keep me going. What an incredible experience knowing you has been.

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ABSTRACT OF THE DISSERTATION

Virulence Mechanisms of Group A *Streptococcus*

by

Anjuli M. Timmer

Doctor of Philosophy in Molecular Pathology
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Group A *Streptococcus* (GAS) is a leading human pathogen infecting millions annually. Colonization is mediated through bacterial attachment to host epithelium. Internalization of epithelial cells may offer the bacteria protection from immune cells and antibiotics, and allow for penetration of epithelial barriers. We investigated the role of surface-anchored protein serum opacity factor (SOF) in the invasion of epithelial cells and virulence. Our data demonstrate that SOF plays a critical role in invasion of epithelial cells, mediated through its activity domain, independent of its fibronectin binding capacity. To this end, elimination

of SOF in GAS produced smaller lesions and yielded significantly less bacteria per lesion than wild-type (WT) in a mouse model of necrotizing fasciitis.

To cause invasive disease GAS must circumvent host immune defenses. Neutrophils circulate in the bloodstream until chemokine signals recruit them to sites of infections. GAS produce a protease, SpyCEP, which cleaves and inactivates one of the most potent neutrophil chemoattractants interleukin-8 (IL-8). We observe that this leads to decreased neutrophil migration compared to a GAS mutant lacking the SpyCEP gene. In addition, we demonstrate that SpyCEP inhibis the formation of neutrophil NETs and contributes to GAS-induced neutrophil apoptosis. The SpyCEP mutant was significantly attenuated for virulence in a mouse model of necrotizing fasciitis.

Macrophages are necessary defenders against GAS infections. We have found that live GAS trigger significant accelerated caspase-dependent macrophage apoptosis upon phagocytosis. This apoptosis disrupts mitochondrial integrity and promotes membrane remodeling, leading to the release of cytochrome *c* into the cytosol. Apoptosis-induction is dependent upon the GAS pore-forming cytolysin streptolysin O (SLO), which is necessary and sufficient for this phenotype. Accelerated apoptosis hampers the ability of macrophages to kill the bacteria and release cytokines, blunting immune activation. An SLO mutant was attenuated in a mouse systemic infection model, with less bacteria surviving in the blood and reduced killing of mice.

This work demonstrates the complexity of GAS interactions with the host during disease and the multitude of virulence mechanisms employed to escape immune defenses. A deeper understanding of GAS disease pathogenesis can lead to novel therapeutic strategies in the treatment of this disease.

Chapter I

Introduction:
Group A *Streptococcus* Immune Interactions

INTRODUCTION

Group A *Streptococcus* (GAS; *Streptococcus pyogenes*) is a chain-forming Gram-positive bacterial pathogen that is responsible for a wide variety of diseases in humans. Clinical manifestations range from superficial, common infections such as pharyngitis (strep throat) and impetigo, to life-threatening invasive conditions such as necrotizing fasciitis (flesh-eating disease) and toxic shock syndrome, or sepsis. Post-infective sequelae including rheumatic heart disease are serious consequences of GAS infections.

It has been conservatively estimated that over 18 million people worldwide currently have serious invasive GAS infections, with almost 2 million new cases each year (Carapetis et al., 2005). Over half a million deaths annually can be attributed to severe GAS infections (Carapetis et al., 2005), highlighting the need for a deeper understanding of GAS infections for the development of novel therapeutic strategies.

There are also estimated to be over 600 million cases of strep throat annually worldwide (Carapetis *et al.*, 2005). A recent study in the United States looked at the economic costs of GAS pharyngitis in school-aged children. By taking into account medical and non-medical costs, they determined that GAS pharyngitis in school children cost the U.S. between a quarter and a half a million dollars annually (Pfoh et al., 2008). A successful vaccine strategy could

appreciably lower this economic burden, as children are thought to be a reservoir in the population for GAS.

Currently greater than 150 serotypes of GAS have been identified. Strains are serotyped based on a variety of factors, primarily *emm* or M type (Johnson et al., 2006). Further classification is based on the presence and/or activity of serum opacity factor (SOF) and the T-antigen, now known to be GAS pili (Mora et al., 2005).

The ability of GAS to cause invasive disease indicates that it is able to resist immune clearance. This review will focus on the mechanisms GAS interactions with the host: inhibition of leukocyte recruitment, evasion of phagocytic killing, novel therapeutic strategies, and vaccine development advances in attempt to reduce the incidence of this disease. Emphasis is placed on recent experimental evidence in these areas.

INHIBITION OF LEUKOCYTE RECRUITMENT

Polymorphonuclear cells (PMNs), or neutrophils, are critical components of the host innate immune system and are frontline defenders against GAS infections. These leukocytes circulate in the blood stream until activating cytokines provoke neutrophil extravasation, where chemokine gradients recruit them to sites of infection. As the name *pyogenes* implies, pus accumulates at the site of GAS skin infections resulting from the degranulation of neutrophils, demonstrating the key role these cells are playing in fighting GAS infections. In a

baboon model of necrotizing fasciitis, animals with high levels of neutrophils at the site of infection survive while those with little neutrophil influx succumb to the infection (Taylor et al., 1999).

The most effective chemoattractants are those that are released by host cells, such as macrophages and endothelial cells, in response to the presence of a pathogen. One of the most potent chemokines for activating and attracting neutrophils to the site of infection is CXCL8, or interleukin-8 (IL-8). GAS expresses a protein, ScpC or SpyCEP, which specifically cleaves IL-8 in the C-terminal region rendering it inactive (Edwards et al., 2005). This cleavage leads to a hampering of neutrophil influx to the site of infection and decreased activation of any neutrophils presents, allowing for enhanced bacterial survival and proliferation. SpyCEP is highly conserved between various GAS serotypes, and has been recently shown to also cleave human chemokines GCP-2 and GROα (Sumby et al., 2008). SpyCEP also cleaves the functional murine homologues of IL-8, KC and MIP-2, although to a lesser degree, representing species specificity (Edwards et al., 2005; Hidalgo-Grass et al., 2006). Recent work by Sumby *et al*. (Sumby et al., 2006) reported that expression of SpyCEP is highly upregulated in strains mutated in the CovR/S regulator and often isolated from infections (see more below).

ScpA is an endopeptidase expressed by GAS that has been reported to cleave another chemokine, C5a (Cleary et al., 1992). C5a is a component of the

complement cascade and is responsible for the recruitment of neutrophils and activation of their bactericidal function against GAS (DeMaster et al., 2002). It has been reported that ScpA works in conjunction with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the cleavage of C5a (Terao et al., 2006). GAPDH can also be released from GAS and bind C5a to form a complex which is then targeted for cleavage by ScpA (Terao et al., 2006).

There have been contradictory reports on the role of SpyCEP in virulence in a mouse model of necrotizing fasciitis. An ScpA mutant and a double mutant of ScpA/SpyCEP were tested in a mouse model of necrotizing fasciitis. No differences were seen between mice infected with WT GAS or the ΔScpA mutant, while the ΔSpyCEP/ScpA double mutant was highly attenuated, forming much smaller lesions and leading to reduced mortality (Hidalgo-Grass et al., 2006). Levels of MIP-2 and KC (murine homologues of IL-8), and numbers of neutrophils, at the sites of infection were significantly higher in the strain lacking SpyCEP than the others, demonstrating the *in vivo* cleavage of these chemokines (Hidalgo-Grass et al., 2006). More recent work reported that a single mutant of SpyCEP was significantly more virulent than the WT in a similar model looking at lesion size in a skin infection model (Sumby et al., 2008). One possible reason given for this discrepancy is the different strains used, the previous one containing a regulatory locus for SpyCEP which the latter one lacks (Sumby et al., 2008).

RESISTANCE TO PHAGOCYTOSIS, COMPLEMENT, AND ANTIBODIES

Apart from inhibiting the chemotaxis of neutrophils, GAS has evolved strategies to evade killing by neutrophils that are present at the site of infection. Inhibition of phagocytosis is a major defense against leukocyte killing. The hyaluronic acid capsule coats the outside of the GAS surface and plays a critical role in the inhibition of phagocytosis (Wessels and Bronze, 1994). The mechanism of this resistance is most likely due to the capsule coating blocking the accessibility of the bacteria to opsonic antibodies. The capsule is also beneficial in cloaking bacterial antigens as it is poorly antigenic itself due to its resemblance to the host. Recent work by Dinkla *et al*. demonstrated that the capsule is highly upregulated upon bacterial passage in blood, and this interferes with the ability of antibodies to G-related α_2-macroglobulin-binding protein (GRAB) to recognize and bind GAS (Dinkla et al., 2007).

The inhibition of the complement pathway is so critical to the pathogenesis of GAS infections that multiple strategies have evolved for evading this host defense mechanism. Activation of the complement pathway results in the deposition of C3b and iC3b on the microbial surface. These are recognized by receptors on leukocytes which phagocytose the bacteria. In order to protect itself, the host has molecules which dampen complement activity, such as factor H (FH) and C4b binding protein (C4BP).

The M protein has been shown to bind regulatory mediators such as FH and C4BP (Horstmann et al., 1988; Thern et al., 1995; Thern et al., 1998; Whitnack and Beachey, 1982) and resist complement-mediated opsonization and phagocytosis (Carlsson et al., 2005). Recent studies have shown that the specific region of M protein that binds to C4BP has significant sequence divergence between strains (Persson et al., 2006), highlighting the need for this protein to retains its antigenic diversity. Courtney *et al*. recently showed that M-related proteins also have the ability to bind fibrinogen, thereby inhibiting complement-mediated phagocytosis (Courtney et al., 2006). This study also observed the first evidence that serum opacity factor (SOF) may play a significant role in the inhibition of phagocytosis by leukocytes (Courtney et al., 2006). SOF is a serotype-specific surface-anchored protein with a fibronectin-binding domain and an activity domain responsible for the opacification of mammalian serum (Jeng et al., 2003). SOF's activity domain has been reported to play a significant role in epithelial cell invasion and virulence in a mouse model of necrotizing fasciitis (Timmer et al., 2006). It has also been reported that another streptococcal fibronectin binding protein, SfbI, shares M protein's ability to resist phagocytosis and complement deposition (Hyland et al., 2007), stressing the importance of this immune evasion strategy for GAS. Streptococcal pyrogenic exotoxin B (speB) has been found to cleave C3 both *in vitro* and *in vivo*, resulting in inhibition of complement-mediated killing and neutrophil recruitment to the site of infection (Terao et al., 2008).

RESISTANCE TO PHAGOCYTIC KILLING

Work in the last few years has focused on the ability of PMNs to ensnare bacteria in neutrophil extracellular traps (NETs), primary made up of DNA and histones (Brinkmann et al., 2004). Bacteria caught in these NETs are then subject to killing by high concentrations of antimicrobial peptides (AMPs) and enzymes (Brinkmann et al., 2004). A major virulence mechanism of GAS is the production of a potent deoxyribonuclease (DNase) (*sda1*) which is able to degrade neutrophil NETs and escape killing (Buchanan et al., 2006). DNases of GAS were shown to be important virulence factors in systemic and skin infection mouse models and a cynomolgus macaque model of pharyngitis (Buchanan et al., 2006; Sumby et al., 2005).

AMPs have potent antimicrobial activities and are released in response to intra- and extracellular pathogens. These small cationic peptides are attracted to the negative charges on bacterial surfaces, where they insert themselves in the membrane and lyse the bacteria. GAS surface protein lipotechoic acid (LTA) is modified by D-alanylation. Kristian *et al*. reported that this modification increases the positive charge of the cell surface, actually repelling AMPs and promoting bacteria survival in the presence of neutrophils (Kristian et al., 2005). D-alanylation of LTA was also demonstrated to be beneficial for epithelial cell adherence and invasion (Kristian et al., 2005).

Nilsson and co-workers recently demonstrated that GAS Factor H and M protein bind to beta-2-glycoprotein I (β_2GPI), inhibiting its cleavage into active AMPs (Nilsson et al., 2008). These factors are also released and inhibit any peptides that were formed by the cleavage of β_2GPI, further protecting GAS (Nilsson et al., 2008). Streptococcal inhibitor of complement (SIC) has also been shown to protect GAS from killing by the enzymatic action of lysozyme (Fernie-King et al., 2002), and the AMPs LL-37 and α-defensin (Frick et al., 2003).

Once inside the cells, GAS has a variety of mechanisms for promoting its own survival. Hakansson *et al*. demonstrated that in epithelial cells, SLO is responsible for preventing internalization of GAS into lysosomes (Hakansson et al., 2005), thereby avoiding killing by the acidic lysosomal environment. M and M-related proteins have been shown in neutrophils to prevent fusion of the phagosome and lysosome, also protecting the bacteria from phagocytic killing (Staali et al., 2006).

The natural life of a neutrophil ends with the activation of cell death programs, or apoptosis, to eliminate these cells in a non-inflammatory way. Another mechanism that GAS uses to avoid killing by neutrophils is to eradicate them through the induction of apoptosis. GAS have been shown to induce rapid and accelerated neutrophil apoptosis compared to other pathogens including *L. monocytogenes* and *S. aureus* (Kobayashi et al., 2003). Recent studies have determined that the pore-forming toxin, streptolysin O (SLO) is responsible for

caspase-mediated induction of macrophage and neutrophil apoptosis (Timmer *et al.*, 2008).

Miyoshi-Akiyama *et al*. found reduced neutrophil recovery from the peritoneal cavity of mice injected with WT GAS compared to a streptolysin S (SLS) mutant (Miyoshi-Akiyama et al., 2005). This was due to killing of the neutrophils as opposed to reduced migration (Miyoshi-Akiyama et al., 2005). Datta *et al*. demonstrated that SLS is produced through a biosynthetic pathway encoded by a nine gene operon, in which each gene is essential for expression of active SLS (Datta et al., 2005). Elimination of SLS production rendered the bacteria more susceptible to neutrophil and human whole blood killing, in addition to attenuation in virulence (Datta et al., 2005).

Nilsson *et al*. reported that SLO formation of pores in the membrane leads to the activation of the p38 MAP kinase pathway through the influx of Ca^{2+} (Nilsson et al., 2006). The Ca^{2+} influx was followed by degranulation of PMNs and the release of heparin binding protein (HBP) and antimicrobial peptides to eliminate the pathogen (Nilsson et al., 2006). SLO has been implicated in cytolysin-mediated translocation (Madden et al., 2001), in which the streptococcal nicotinamide dehydrogenase (NADase) is specifically delivered into the cytosol of cells through SLO pores (Ghosh and Caparon, 2006; Madden et al., 2001). The NADase is a virulence factor which enhances the cytotoxic effects of SLO through the depletion of host cell energy stores (Michos et al., 2006).

Lung injury was recently found to be caused by the degranulation of PMNs, triggered by M protein, and this resulted in increased vascular permeability (Soehnlein et al., 2008).

Coagulation dysregulation is a common symptom of sepsis and several GAS proteins are reported to induce pro-coagulatory effects. The major effector of this phenotype is the M protein which triggers coagulation through the upregulation of tissue factor on monocytes (Pahlman et al., 2007), and induces platelet activation and thrombus formation by binding fibrinogen and forming complexes including IgG (Shannon et al., 2007). These platelets activate neutrophils and macrophages leading to further coagulation and inflammation (Shannon et al., 2007). Purified streptolysin O has also been found to induce coaggregation of platelets and neutrophils (Bryant et al., 2005).

IMMUNE ACTIVATION

Sepsis is due to an uncontrolled inflammatory immune response to a bacterial infection. There is a fine balance between adequate immune activation to sufficiently alert the immune system of an invading pathogen and recruit the immune cells to clear the infection, and an over-stimulation of the immune response that is detrimental to the host. GAS is a leading cause of toxic shock syndrome (TSS), which is primarily mediated through the expression of superantigens. Superantigens have the ability to activate T cells in a non-antigen specific manner, leading to an uncontrolled immune response, resulting in multi-

organ failure. By binding to both MHC II and T cell receptors, superantigens can evoke a T cell response that is more potent than a normal antigen (Sriskandan et al., 2007). The purpose of the expression of superantigens by GAS is not well understood, but neutralization of superantigens could be an effective therapy to treat streptococcal TSS.

On initial contact with the host, GAS stimulates epithelial cells to release a host of cytokines, including IL-6 and IL-8, through activation of the NF-κB and MAP kinase signaling pathways (Tsai et al., 2006). M protein stimulation of epithelial cells leads to the release of chemokines CXCL9, -10, and -11, which are produced at bactericidal levels (Egesten et al., 2007; Eliasson et al., 2007). SIC is able to inhibit their antimicrobial activities, but not their chemokine functions (Egesten et al., 2007).

Toll-like receptors (TLRs) are receptors that specifically recognize pathogen-associated molecular patterns, or PAMPs, and then signal an inflammatory reaction. M protein has also been found to interact with Toll-like receptor 2 (TLR2) on monocytes, leading to the release of inflammatory cytokines (Pahlman et al., 2006). This response is enhanced by the presence of heparin binding protein (Pahlman et al., 2006), whose release from neutrophils is triggered by SLO pore formation (Nilsson et al., 2006).

T cells are members of the lymphocyte family and are responsible for cell-mediated immunity. M protein has been reported to have diverse effects on T cells. Price *et al*. reported that M protein interacts with CD46 on CD^{4+} T cells and induces a regulatory, non-proliferative, anti-inflammatory phenotype, with high levels of immunosuppressive IL-10 (Price et al., 2005). Pahlman and colleagues found that M protein incubation with PBMCs activated T cell proliferation and the release of inflammatory cytokines (Pahlman et al., 2008). This demonstrates the balance that is occurring between inflammation and non-inflammatory signaling during GAS infections.

Dendritic cells (DCs) are professional antigen presenting cells which may be some of the first cells to encounter GAS in the host. Quite a bit of recent work has focused on the interactions of GAS with dendritic cells and found that DC maturation (as measured by the expression of cell surface markers) is triggered by GAS infection (Loof et al., 2007). GAS have been found to activate both myeloid and plasmacytoid DCs (Veckman and Julkunen, 2008) through a MyD88-dependent mechanism (Loof et al., 2008). Interestingly, MyD88 was found to be dispensable for internalization and killing of GAS by DCs (Loof et al., 2008). Depletion of DCs in a mouse model increased bacterial loads during infection, which was due to hampered IL-12 release (Loof et al., 2007), a cytokine which has been shown to play a critical role in the clearance of GAS infections (Metzger et al., 1995).

SWITCH TO INVASIVE PHENOTYPE

Recent experimental observations have found that there is an inverse correlation between invasive disease GAS isolates and the expression of speB, a cysteine protease that degrades many streptococcal proteins (Cole et al., 2006). This could be explained by the fact that speB interferes with the accumulation of plasmin activity on the GAS surface, which has been found to be critical for GAS invasive disease (Cole et al., 2006). Sumby *et al*. also noticed significant differences in gene expression between pharyngitis strains and invasive disease isolates, linking the differences to a mutation in the covR/S two-component regulatory system of GAS (Sumby et al., 2006). CovR/S is responsible for influencing approximately 15% of all GAS genes, including repression of major virulence factors such as the hyaluronic acid capsule, DNase, streptokinase, and streptolysin S, as well as other regulators (Federle et al., 1999; Graham et al., 2002). Walker *et al*. went on to determine that upregulation of DNase expression in the covR/S mutant is the selective pressure for this mutation in invasive disease (Walker et al., 2007), highlighting the importance of evading killing by PMN NETs in establishing invasive infection.

NOVEL THERAPEUTIC AND VACCINE STRATEGIES

New research into the virulence mechanisms involved in GAS interactions with the immune system can lead to the design of novel therapies to target these factors. Buchanan *et al*. demonstrated that the use of G-actin to inhibit the streptococcal DNase was an effective therapy in a mouse model of NF

(Buchanan et al., 2006). Soehnlein and colleagues suggest inhibition of the degranulation of neutrophils triggered by M protein as a therapy to hinder lung damage (Soehnlein et al., 2008).

Another novel therapy involves a bacterial pheromone peptide, streptococcal invasion locus CR (SilCR), a regulatory peptide which exerts an inhibitory effect on the IL-8 protease SpyCEP (Hidalgo-Grass et al., 2004). Studies have shown that administering the SilCR peptide to mice allows for increased neutrophil migration to the site of infection and enhances clearance of the bacteria (Hidalgo-Grass et al., 2004). This therapeutic strategy is not directly antimicrobial but allows for augmentation of our immune system's ability to fight the infection.

The elimination of neutrophils and macrophages through the induction of apoptosis by GAS can also lead to increased bacterial survival. Experiments inhibiting the apoptosis of macrophages infected with GAS showed enhanced killing of the bacteria in comparison to untreated macrophage (Timmer *et al.*, 2008). This is another example of a therapy which takes advantage of our natural ability to fight infection.

Vaccinations for GAS have been researched for decades, but there are still no commercially available vaccines. The majority of vaccination strategies have focused on the M protein. This protein is highly antigenic, and while there

have been quite a few promising studies done using this protein, there are some drawbacks. The first disadvantage is that M proteins have regions that are cross-reactive to self and therefore immunization with M protein could lead to self-reactive antibodies being generated, as occurs with rheumatic heart disease. The second complication in developing an M protein vaccine is the variability. As discussed before, there are over a hundred different serotypes of GAS based on M proteins, and antigenic variation in this critical virulence mechanism permits a person to be repetitively infected with multiple GAS strains.

Due to the factors described above in regards to using M protein as a vaccine candidate, much research has been done in identifying peptides of M protein from the more conserved C-terminal region which do not cross-react with heart muscle antibodies. Vohra *et al*. identified a peptide sequence that is often repeated in some M proteins and offered protection from more than 30 strains tested (Vohra et al., 2005). The more copies of this peptide the strain had, the better the protection. Cell-mediated autoimmunity was avoided as this peptide had a B cell epitope, but no T cell epitope (Vohra et al., 2005). Additional studies have been done using conserved M protein peptides in conjunction with peptides from the fibronectin binding repeats of SfbI and various adjuvants (Olive et al., 2007; Schulze et al., 2006), demonstrating that multiple peptides offered more protection than one.

A fascinating study by McNamara *et al*. elucidated the protein structure of the N-terminal variable region of the M protein from the highly invasive clinical M1T1 strain. It was determined that the irregular coiled coils of the M protein resemble the heart proteins myosin and tropomyosin (McNamara et al., 2008), possibly providing an explanation for the cross-reactivity of M protein antibodies with host tissues. Amino acid substitutions in the M1 protein to promote coiled coil stability lead to a loss of fibrinogen binding capacity, reduced proinflammatory effects such as the release of HBP, and diminished antibody cross-reactivity, while still retaining full protection as a vaccine (McNamara et al., 2008). This idealized stable form of M1 protein could serve as a superior vaccine candidate considering that even with the upregulation of capsule masking other antigens after passage in human blood, the N-terminal portion of M protein is still accessible to antibody recognition (Dinkla et al., 2007).

Although there have been many exciting breakthroughs in M protein-based vaccine strategies, due to the various complications with the use of M protein, many recent studies have focused on other molecules to protect against GAS infection. Successful studies in the past few years include vaccination with the pilus proteins (Mora et al., 2005), fibronectin binding protein A (FbaA) which is not expressed in all strains (Terao et al., 2005), streptococcal immunoglobulin binding protein 35 (Sib35) (Okamoto et al., 2005), and SOF (Gillen et al., 2008), both of which are found in all strains.

CONCLUSIONS

An increase in the incidence of invasive GAS diseases demonstrates the ability of this pathogen to breach host barriers and evade innate host defenses. New studies in the differences between invasive and non-invasive infections have brought insight into the rapid evolution that occurs in the bacteria during a single infection. This change in the bacterial phenotype alters the balance between the pathogen and host and results in a much more serious invasive infection.

As discussed here, GAS possess a vast array of mechanisms to resist immune clearance at multiple stages of infection. These include the inhibition of neutrophil recruitment, resistance to phagocytosis and complement deposition, and defense against killing mechanisms such as NETs and AMPs. The M protein has been found to be involved in many of these strategies and therefore has potential as a vaccine candidate, with exciting recent research focusing on ways to avoid the complications of antigenic variability and antibody cross-reactivity.

The following chapters will discuss work I have done investigating three virulence mechanisms using molecular Koch's postulates and infection models of GAS disease. This work demonstrates serum opacity factor's role in epithelial cell invasion and skin infections, SpyCEP's degradation of IL-8 resulting in impaired neutrophil recruitment and activation, and streptolysin O induction of

macrophage apoptosis to enhance bacterial survival and blunt activation of the immune system.

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Chapter II

Serum Opacity Factor Promotes Group A Streptococcal Epithelial Cell Invasion and Virulence

ABSTRACT

Serum opacity factor (SOF) is a bifunctional cell surface protein expressed by 40-50% of group A streptococcal (GAS) strains, comprised of a C-terminal domain that binds fibronectin and an N-terminal domain that mediates opacification of mammalian sera. The *sof* gene was recently discovered to be co-transcribed in a two-gene operon with a gene encoding another fibronectin-binding protein, *sfb*X. We compared the ability of an SOF(+) wild-type (WT) serotype M49 GAS strain and isogenic mutants lacking SOF or SfbX to invade cultured HEp-2 human pharyngeal epithelial cells. Elimination of SOF led to a significant decrease in HEp-2 intracellular invasion while loss of SfbX had minimal effect. The hypoinvasive phenotype of the SOF(-) mutant could be restored upon complementation with the *sof* gene on a plasmid vector, and heterologous expression of *sof*49 in M1 GAS or *Lactococcus lactis* conferred marked increases in HEp-2 cell invasion. Studies using a mutant *sof*49 gene lacking the fibronectin-binding domain indicated that the N-terminal opacification domain of SOF contributes to HEp-2 invasion independent of the C-terminal fibronectin binding domain, findings corroborated by observations that a purified SOF N-terminal peptide could promote latex bead adherence to HEp-2 cells and inhibit GAS invasion of HEp-2 cells in a dose-dependent manner. Finally, the first *in vivo* studies to employ a single gene allelic replacement mutant of SOF demonstrate that this protein contributes to GAS virulence in a murine model of necrotizing skin infection.

INTRODUCTION

Group A *Streptococcus* (GAS) is responsible for a wide spectrum of human diseases, from simple pharyngitis to serious invasive infections including necrotizing fasciitis and toxic shock syndrome. Approximately half of GAS strains are capable of opacifying mammalian serum, a unique phenotype attributable to the presence of serum opacity factor (SOF) encoded by the *sof* gene (Beall *et al.*, 2000; Kreikemeyer *et al.*, 1995). SOF is a 110-kDa protein with an LP(X)SG cell wall anchor motif expressed in both membrane bound and extracellular forms (Courtney *et al.*, 1999; Katerov *et al.*, 2000; Rakonjac *et al.*, 1995). SOF is a bifunctional protein comprised of an N-terminal opacification domain, and C-terminal domain of highly conserved tandem repeat sequences capable of binding the host extracellular matrix components fibronectin and fibrinogen (Courtney *et al.*, 2002; Kreikemeyer *et al.*, 1999; Rakonjac *et al.*, 1995). Once thought to be a lipoproteinase, SOF has recently been shown to bind apoA-I and apoA-II, causing release of HDL lipid cargo, which in turn coalesces to form lipid droplets, resulting in serum opacification (Courtney *et al.*, 2006b).

Studies with SOF-coated latex beads indicate the protein can promote adherence to human HEp-2 pharyngeal epithelial cells including fibronectin-specific interactions (Oehmcke *et al.*, 2004). A role in GAS virulence has been attributed to SOF, since insertional inactivation of the *sof* gene in a serotype 2 GAS isolate reduced mortality in a mouse intraperitoneal infection model (Courtney *et al.*, 1999). SOF also triggers host production of opsonic antibodies

that protect against infection by SOF(+) strains of GAS, suggesting it may represent a useful vaccine antigen (Courtney *et al.*, 2003).

The *sof* gene was recently recognized to be a member of a two gene operon, invariably co-transcribed with a gene encoding a second surface anchored protein, *sfb*X (Jeng *et al.*, 2003). SfbX possesses a C-terminal domain of tandem repeat sequences very highly homologous to the fibronectin-binding domain of SOF, but a unique N-terminal domain. SfbX is capable of binding fibronectin, but the function of its N-terminal sequences has yet to be elucidated (Jeng *et al.*, 2003). Given previous insertional mutagenesis studies targeting *sof* were performed before the operon structure was recognized, polar effects on *sfb*X would be anticipated, and the precise contribution of each individual gene is therefore uncertain.

In this study, we utilize nonpolar allelic replacement mutants of *sof* and *sfb*X, coupled with heterologous expression of each gene, to study their contribution(s) to GAS epithelial cell invasion and animal virulence. Unexpectedly, our results uncovered a key contribution of the N-terminal SOF opacification domain to GAS cellular invasion independent of the C-terminal fibronectin-binding motifs.

RESULTS

SOF and not SfbX contributes to epithelial cell invasion by M49 GAS. Allelic replacement mutants of M49 GAS lacking SOF or SfbX were compared to the WT parent strain for their ability to invade cultured monolayers of HEp-2 human pharyngeal epithelial cells in vitro. In the graphical representation of our results, photo insets depicting the serum opacity phenotype of the individual bacterial strains are provided within the corresponding bars. As measured by antibiotic protection assay, the isogenic ΔSOF mutant exhibited a 50% decrease in cellular invasion ($P = 0.0002$). The invasion phenotype of the ΔSOF mutant could be restored above WT levels by return of the single *sof* gene on a multicopy number complementation plasmid ($P < 0.0001$) (**Fig. 2.1**). The isogenic ΔSfbX mutant did not differ significantly from WT GAS in HEp-2 cell invasiveness ($P = 0.255$) (**Fig. 2.1**). No difference in overall adherence of WT and ΔSOF mutant GAS was observed, suggesting the contribution of SOF to HEp-2 cell invasion was not simply secondary to increased bacteria–epithelial cell interaction (**Fig. 2.2**). Furthermore, as SOF and SfbX share highly homologous C-terminal fibronectin-binding repeat domains, these results provided a first clue that N-terminal sequences in the “opacification” domain of SOF could possess unique properties facilitating cellular invasion.

Gain of function analysis by heterologous expression of SOF and SfbX. To determine if *sof* and *sfbX* are sufficient to confer increased invasion of

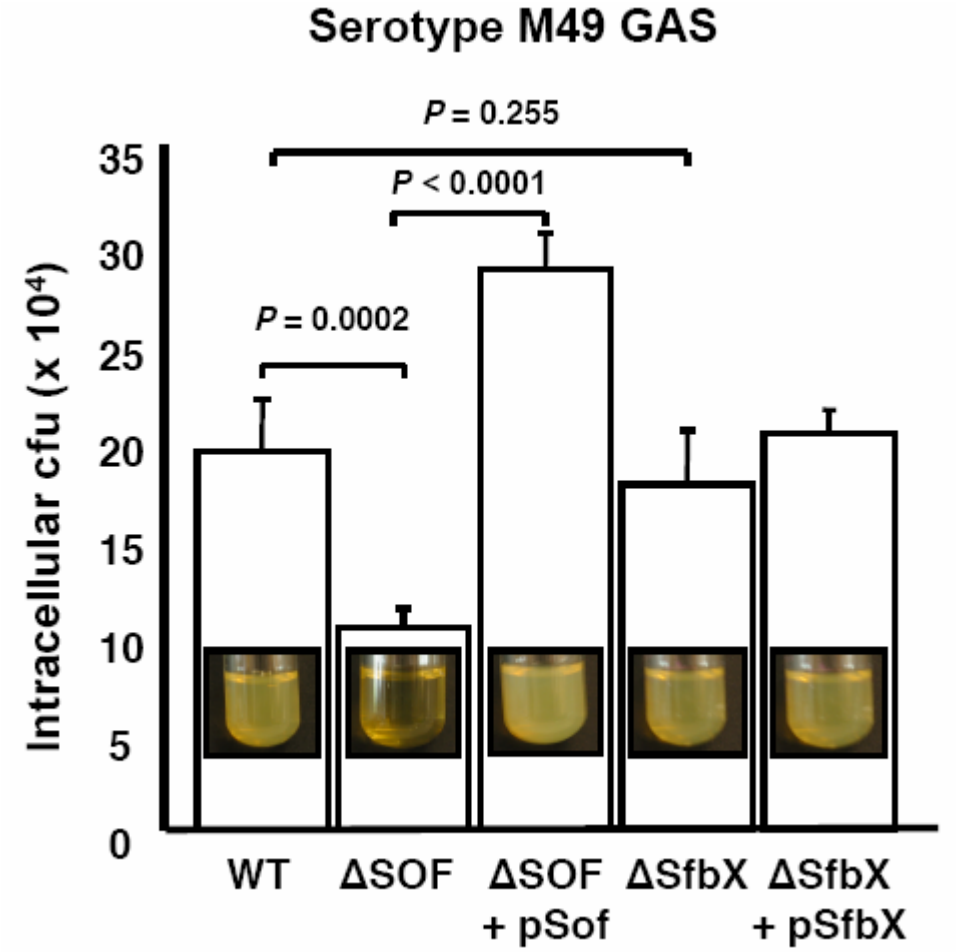

Figure 2.1 Invasion of HEp-2 human pharyngeal epithelial cells by WT M49 GAS, isogenic allelic replacement mutants lacking either SOF or SfbX, and each mutant complemented by replacement of the corresponding gene on a plasmid vector. Figure insets show serum opacification phenotype of each tested strain.

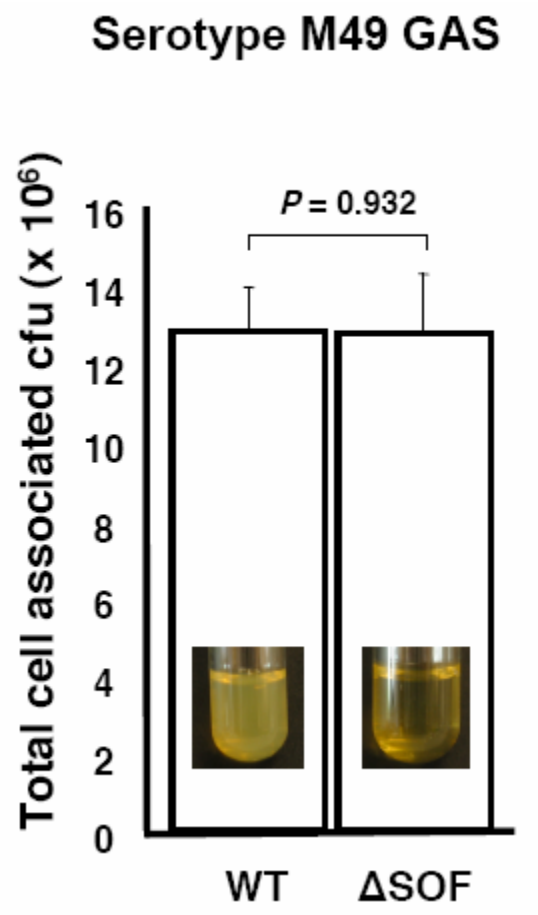

Figure 2.2 Adherence toHEp-2 cells of WT M49 GAS stain and the isogenic ΔSOF mutant.

epithelial cells, expression vectors for each GAS M49 gene were transformed into bacteria lacking this operon. Under the standard assay condition, overexpression of *sof49* in GAS M1 strain 5448 increased Hep2-cell invasion 100-fold, while overexpression of sfbX produced a 10-fold gain in invasion frequency (**Fig. 2.3A**). When parallel studies of heterologous expression were performed in *L. lactis*, which lacks significant fibronectin binding, *sof* conferred a nearly 1,000-fold increase in HEp-2 cell invasion compared to a 10-fold increase for *sfb*X (**Fig. 2.3B**). These analyses in heterologous bacterial strains are also consistent with the key role of SOF, but not SfbX, in epithelial cell invasion by GAS (**Fig. 2.1**).

Visualization of SOF-dependent bacterial uptake in HEp-2 cells. To corroborate observed differences in HEp-2 cell invasion detected in the antibiotic protection assays, fluorescence techniques were used to differentiate surface bound and intracellular bacteria. Bacteria were stained with calcein-AM (green) prior to use in a standard HEp-2 cell invasion assay as above, but at the assay end point the fluorescence of external bacteria was quenched with ethidium bromide (red). As seen in **Fig. 2.4**, noticeably fewer ΔSOF mutant GAS were observed inside HEp-2 cells compared to the WT parent strain. Likewise, the number of M1 GAS and *L. lactis* internalized by HEp-2 cells were markedly increased when these bacteria expressed the cloned M49 *sof* gene (**Fig. 2.4**).

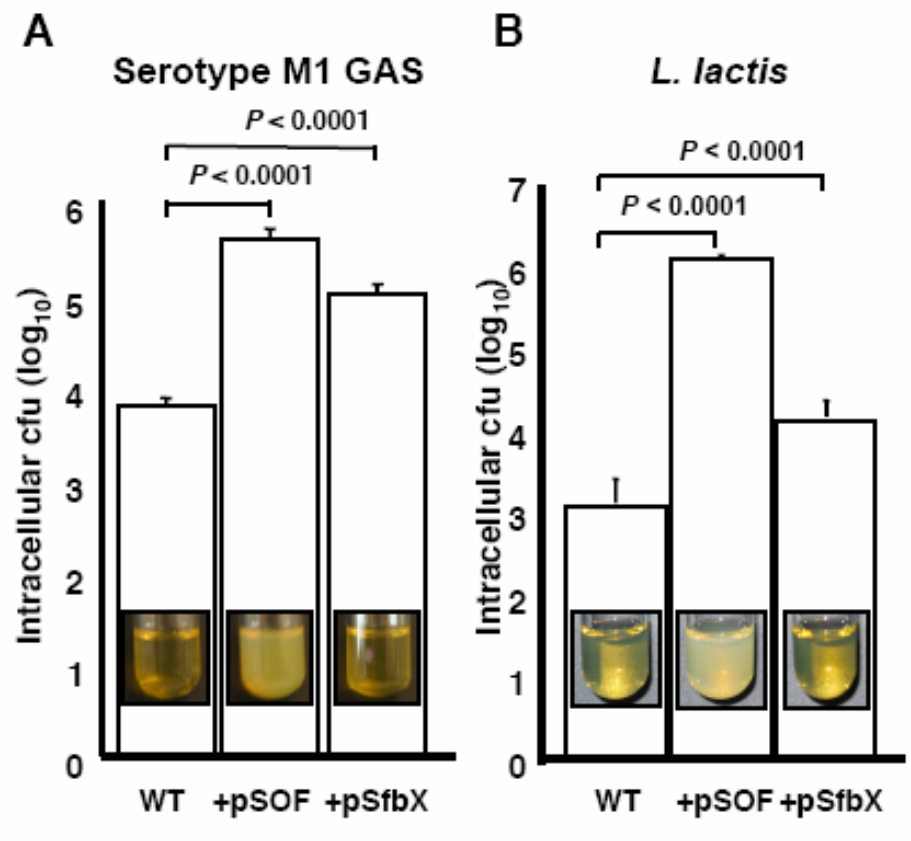

Figure 2.3 Invasion of HEp-2 human pharyngeal epithelial cells upon heterologous expression of the *sof* and *sfb*X genes from M49 GAS in (a) M1 GAS that normally lacks the corresponding operon and (b) *Lactococcus lactis* strain NZ9000. Figure insets show serum opacification phenotype of each tested strain.

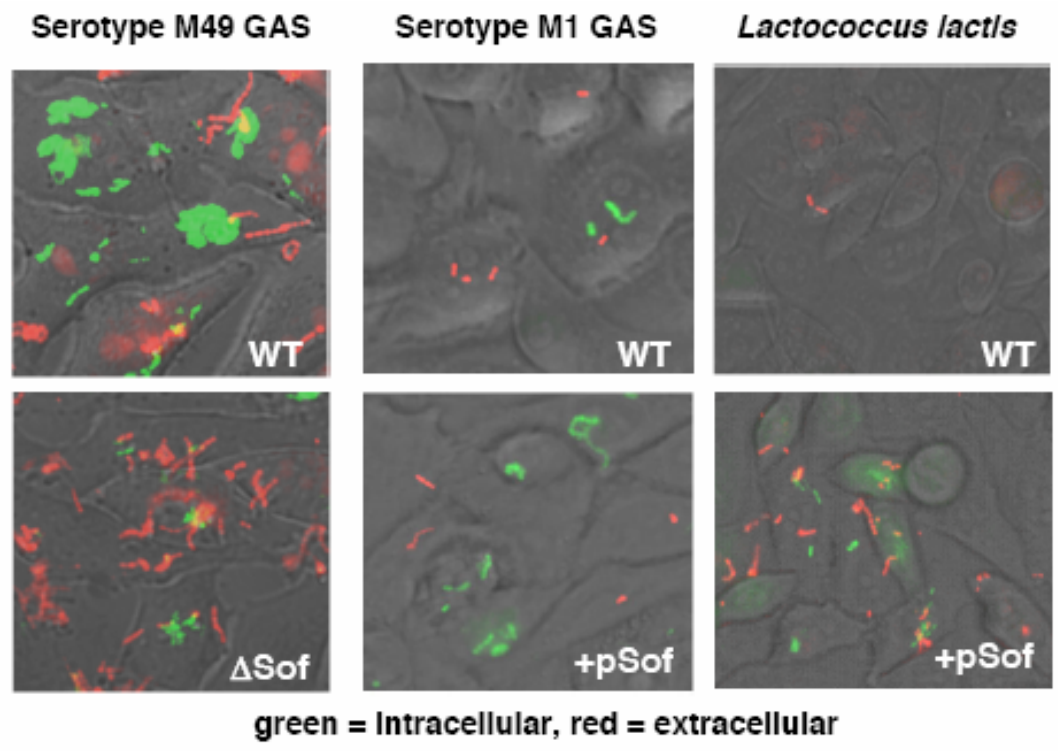

Figure 2.4 Fluorescent imaging demonstrating the contribution of SOF to interactions with HEp-2 epithelial cell monolayers. Green fluorescence represents the intracellular bacteria, and red fluorescence is the extracellular bacteria. Decreased intracellular invasion is seen upon deletion of SOF from M49 WT SOF(+) GAS and increased intracellular invasion observed upon heterologous expression of SOF in M1 WT SOF(-) GAS and *Lactococcus lactis*.

The N-terminal enzymatic domain of SOF contributes to epithelial cell invasion independently of the C-terminal fibronectin-binding domain. We observed in our mutagenesis and heterologous expression studies that SOF contributed much more significantly than SfbX to epithelial cell invasion (**Figs. 2.1, 2.3**), though both proteins possess homologous C-terminal fibronectin-binding domains. We hypothesized that the N-terminal enzymatic domain of SOF must therefore play a direct role in promoting epithelial cell invasion. To test this hypothesis, we generated an in-frame deletion of the entire fibronectin-binding repeat domain of SOF within our expression plasmid (pAT-SOFΔFn) to produce a truncated derivative of the protein that maintained opacification activity. Plasmid pAT-SOFΔFn expressing the truncated SOFΔFn protein was observed to complement the invasion defect of the M49 NZ131Δ*sof* mutant to WT levels (**Fig. 2.5A**), and to confer a 100-fold increase in epithelial cell invasion when expressed in *L. lactis* (**Fig. 2.5B**).

To corroborate these findings, the opacification domain of GAS SOF75, lacking the fibronectin-binding repeats, was expressed in *E. coli* and purified (SOF75ΔFn). Addition of the exogenous SOF75ΔFn peptide produced a dose-dependent inhibition of HEp-2 cell invasion by a WT SOF(+) M75 GAS strain (**Fig. 2.6**), consistent with competitive inhibition of a GAS-host cell interaction mediated by surface-bound SOF. When complexed to latex beads, the same SOF75ΔFn peptide promoted tight adherence of the beads to HEp-2 cells as

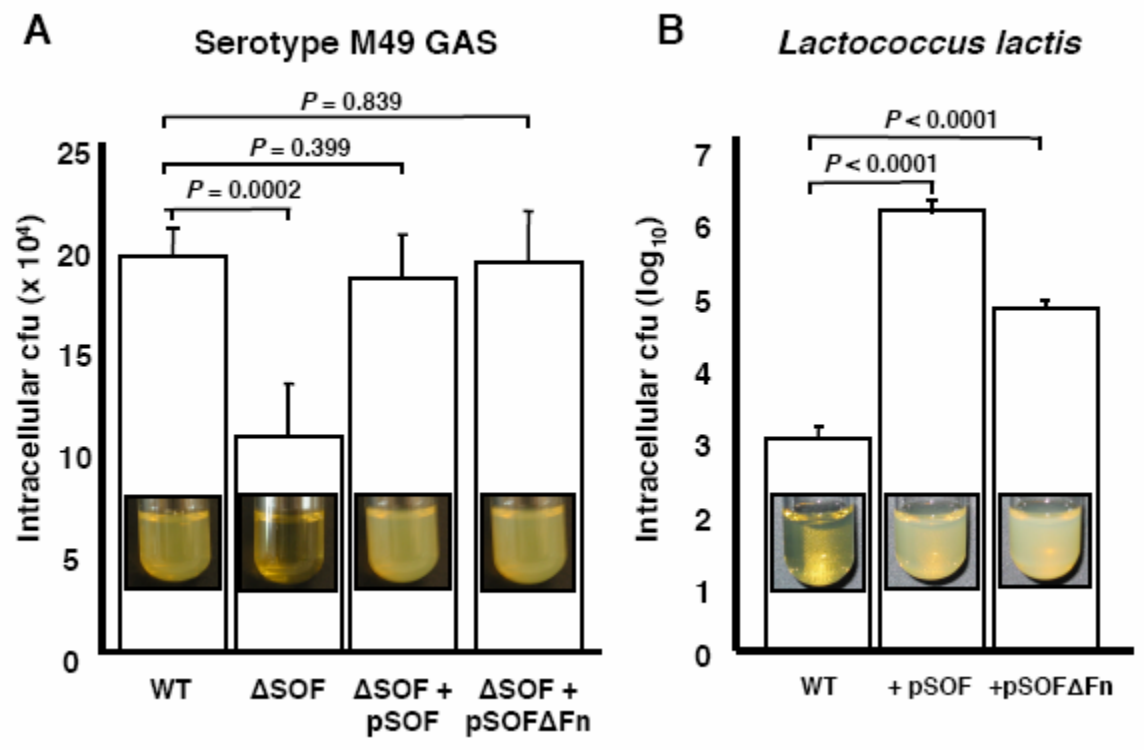

Figure 2.5 Deletion of the fibronectin-binding domain of SOF protein does not eliminate the contribution of SOF to HEp-2 epithelial cell invasion as tested in (A) complementation studies using the M49 GAS ΔSOF mutant and (B) heterologous expression studies in *Lactococcus lactis*. Figure insets show serum opacification phenotype of each tested strain.

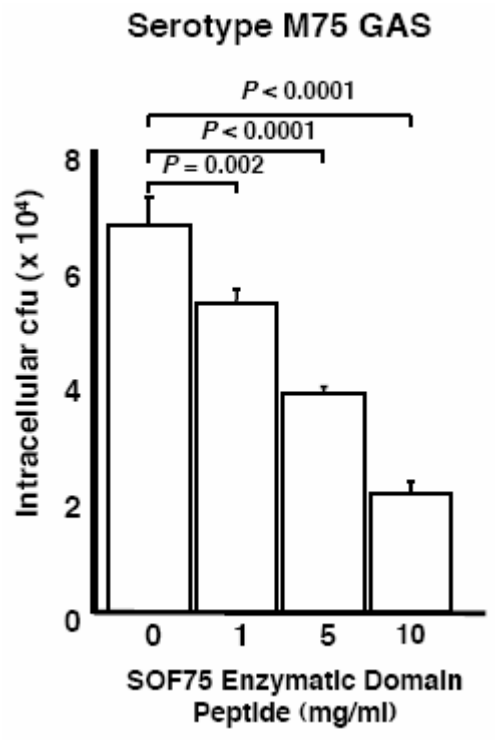

Figure 2.6 An SOF peptide lacking the fibronectin-binding repeats produces a dose-dependent inhibition of HEp-2 epithelial cell invasion by WT SOF(+) GAS.

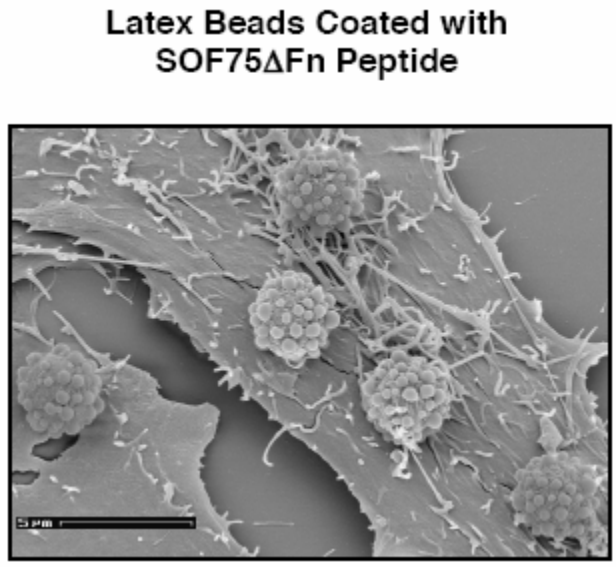

Figure 2.7 An SOF peptide lacking fibronectin-binding repeats promotes adherence of latex beads to HEp-2 cells.

visualized by scanning electron microscopy (**Fig. 2.7**). Taken together, these results indicate that the N-terminal opacification domain of SOF contributes to epithelial cell invasion independently of the C-terminal fibronectin-binding domain.

Correlation of SOF with HEp-2 cell invasiveness among GAS strains. To ascertain whether the observed contribution of SOF to GAS invasiveness in our molecular genetic studies could be reflected broadly across strains of varying SOF phenotype, we studied a panel of GAS strains of various *emm* (M protein) genotypes from the Centers for Disease Control including both SOF(+) (n = 26) and SOF(-) (n = 19) types. These strains were screened for invasion of HEp-2 cells in a 96-well format modification of the standard assay (**Fig. 2.8**). Mean intracellular cfu recovered from the SOF(+) strains was 130,274 compared to 50,233 for the SOF(-) strains; median values were 55,150 for the SOF(+) strains and 29,700 for the SOF(-) strains. A significant positive correlation was thus apparent between GAS expression of SOF and invasiveness in HEp-2 epithelial cells ($P = 0.025$).

SOF contributes to GAS virulence in murine infection. The specific contribution of SOF to GAS virulence was tested in murine models of infection. In **Fig. 2.9**, Kaplan-Meyer survival curves were established for groups of 10 male CD1 mice challenged by intraperitoneal (I.P.) injection with 2×10^7 cfu of either the WT M49 GAS strain or its isogenic ΔSOF and ΔSfbX mutants. No difference

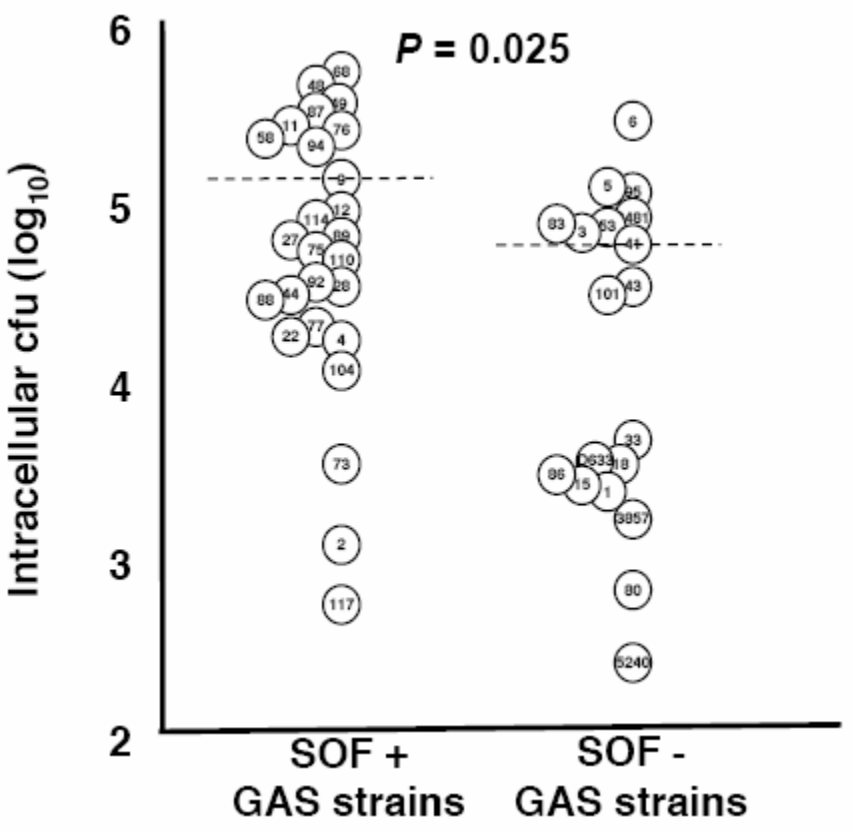

Figure 2.8 Survey of panel of 45 GAS strains of varying M-protein (*emm*) genotypes reveals a correlation of SOF-positivism with increased capacity for HEp-2 cell invasion.

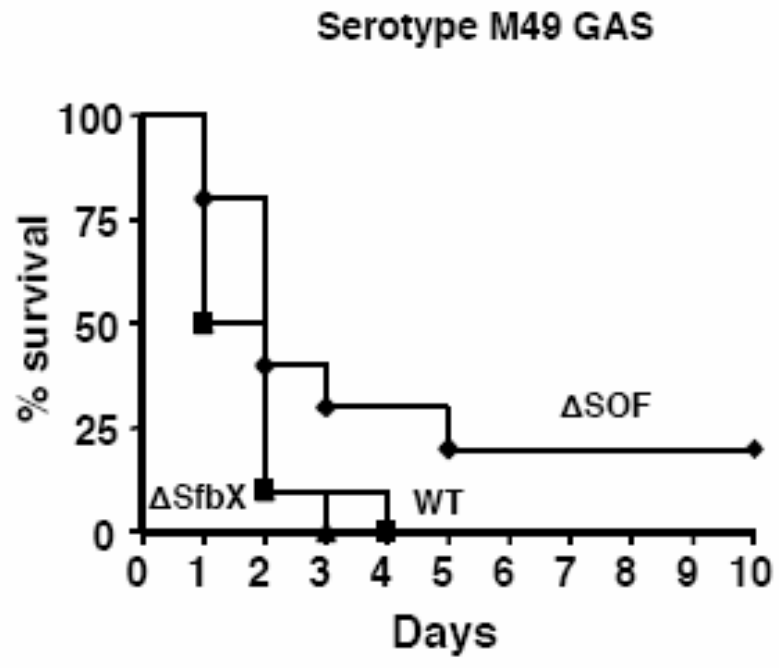

Figure 2.9 Effect of deletion of SOF or SfbX on mouse survival in an intraperitoneal challenge model using an SOF(+) M49 parent strain.

was seen between the WT strain and the ΔSfbX mutant, with 100% mortality by day 3-4; a slight delay in the kinetics of killing of the ΔSOF mutant was observed with 25% of mice surviving and asymptomatic at the end of the 10 d period. Groups of 12 mice injected with SOF expressed heterologously in the WT M1 GAS strains were compared to vector only control using an I.P. challenge dose of 2 x 10^6 cfu. An increase in mortality from 50% to 75% was observed by 10 d (**Fig. 2.10**). As the observed tendencies following loss or gain of SOF did not reach statistical significance, we conducted a further assessment of the contribution of SOF to GAS virulence in a murine subcutaneous infection model. Sixteen male CD1 mice were shaved and injected subcutaneously in one flank with 1 x 10^8 cfu WT GAS M49 strain and in the contralateral flank with an equivalent dose of the isogenic ΔSOF mutant, each mouse serving as its own control. The development of necrotic ulcers was monitored and lesion area calculated at day 7 (**Fig. 2.11A**). Lesions produced by the WT strain were larger in 12/16 mice, lesions equivalent in size in 3/16 mice, and in only 1/16 mice was the lesion produced by the ΔSOF mutant larger. Photos of two representative animals from the skin challenge experiment are shown in **Fig. 2.11B**. The lesions were excised and cfu/gm skin tissue calculated by plating dilutions of tissue homogenate. Bacterial counts were significantly (P = 0.002) higher for the WT strain compared to the ΔSOF mutant in 14/16 mice, with a median 7.7-fold increase of cfu/gm tissue recovered for the WT strain (**Fig. 2.12**). Our results confirm a unique contribution of SOF to GAS virulence independent of SfbX and are the first to demonstrate a role for SOF in GAS pathogenicity using a localized infection model.

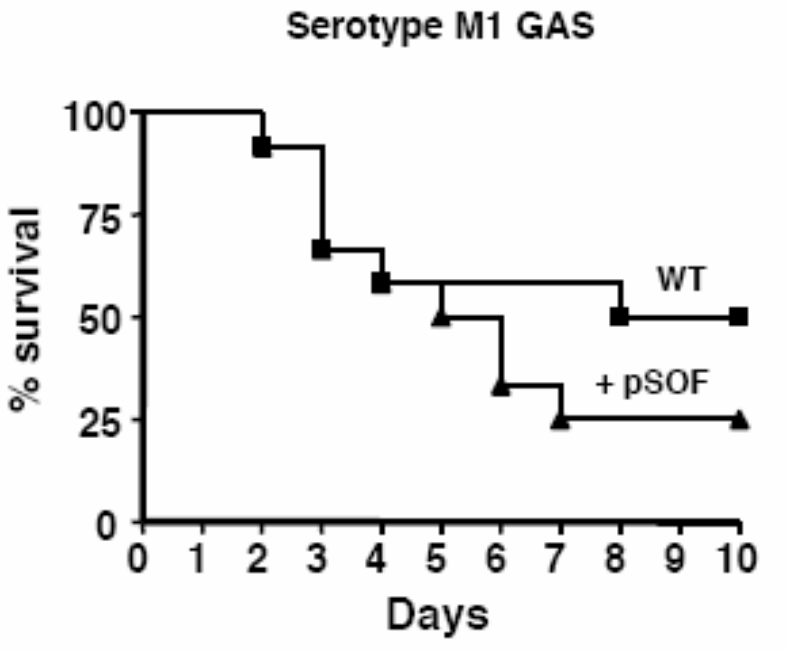

Figure 2.10 Effect of heterologous expression of SOF in the SOF(-) M1 GAS background on mouse survival in and intraperitoneal challenge model.

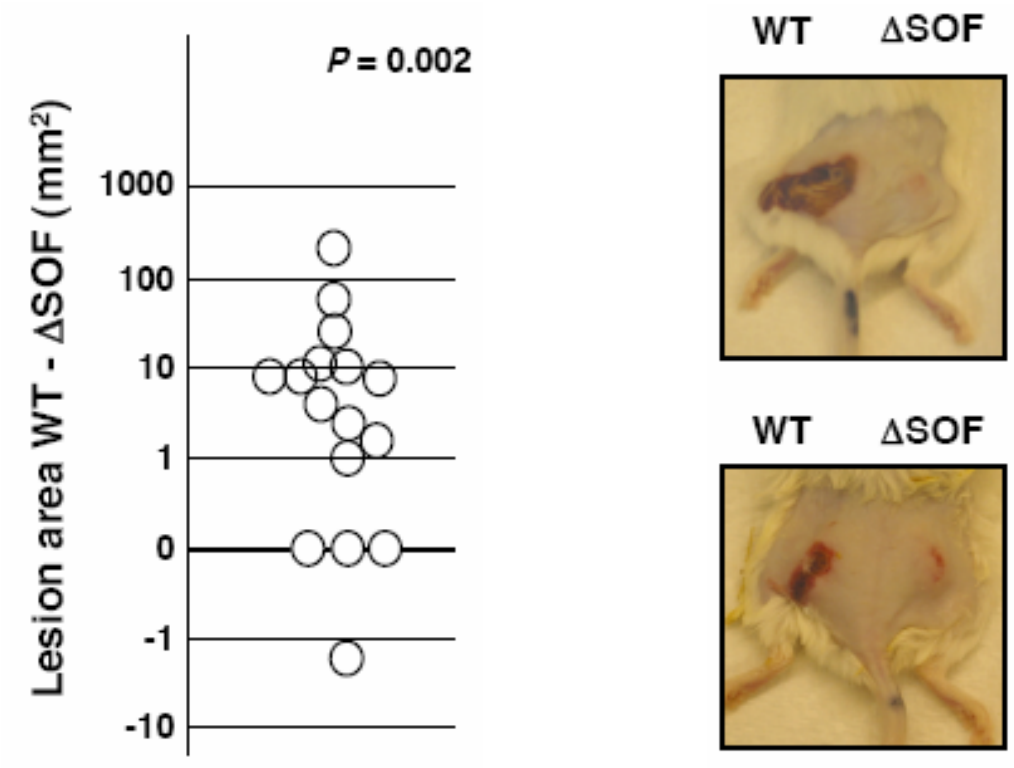

Figure 2.11 Effect of deletion of SOF on GAS virulence in a murine skin infection model, including (A) decreased lesion size (represented by difference in lesion size) (B) representative images of necrotic lesions developing in animals infected using this model.

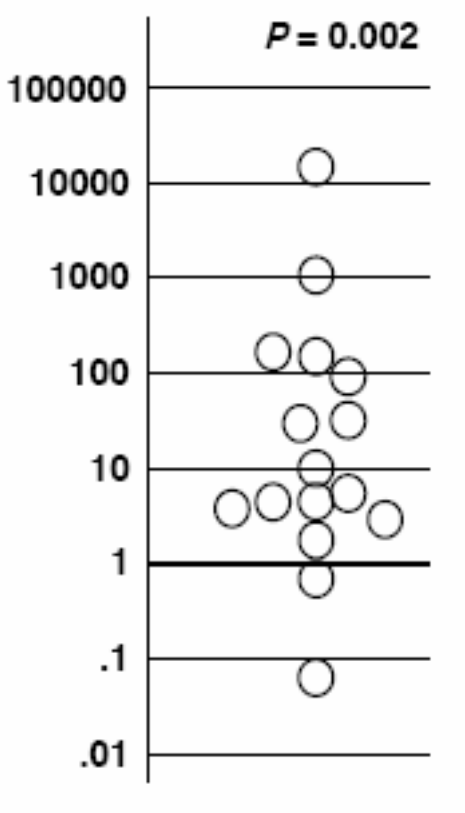

Figure 2.13 Decreased M49 ΔSOF bacterial counts compared to WT M49 in the infected skin tissues (represented by ratio of WT bacterial counts divided by ΔSOF counts).

DISCUSSION

SOF is a unique bifunctional protein produced by many GAS strains and possesses roles in host cell interaction, immunogenicity, and disease pathogenesis. Here we have used isogenic mutants and heterologous expression to show for the first time that SOF promotes GAS invasion of human pharyngeal epithelial cells and contributes to GAS virulence independent of the co-expressed SfbX protein.

The affinity of several GAS surface anchored proteins to fibronectin and other extracellular matrix components has been shown to play a major role in the organism's adherence to and invasion of host epithelial cell surfaces (Hanski and Caparon, 1992; Kreikemeyer *et al.*, 1995; Molinari *et al.*, 1997; Rocha and Fischetti, 1999). Our experiments with a domain deletion construct and purified protein reagent demonstrate that the N-terminal enzymatic domain of SOF, in addition to its activity in serum opacification, possesses distinct pro-invasive properties. The N-terminal domain of SOF thus may cooperate with the C-terminal fibronectin-binding repeats in promoting GAS-host cell interactions. We found SfbX, with homologous C-terminal repeats to SOF, can confer increased invasion in the *L. lactis* background where fibronectin-binding is otherwise absent. However, deletion of *sfb*X was insufficient to impair invasion

of GAS, where the presence of additional fibronectin-binding surface molecules may provide compensation.

The *sof*/*sfb*X operon is located approximately 10 kb from the tandemly-linked genes of the GAS *mga* regulon on a 5,500 bp fragment that appears in *sof* positive strains as a precise insertion relative to the same chromosomal region of *sof*-negative M1, M3, and M18 strains (Jeng *et al.*, 2003). There is a strict correlation of the presence of *sof* with specific *emm* sequence types. It is interesting that this strict correlation is observed, even though many *emm* types are shared by *sof*-positive strains that have unrelated *sof* genes and multilocus sequence types (Beall *et al.*, 2000). Expression of *sof/sfbX* is influenced by the multiple gene regulator (*mga*) that lies closely upstream of *emm* and controls several GAS virulence genes involved in colonization, cellular invasion and immune resistance (McIver and Myles, 2002); *mga* has recently been shown to bind to a specific promoter element upstream of *sof*/*sfb*X in a fashion sufficient to activate transcription (Almengor *et al.*, 2006). We found specific deletion of SOF in the M49 GAS background significantly reduced virulence in the mouse model of necrotizing skin infection. The importance of SOF in this model may reflect contributions not only to host cell adherence and intracellular invasion, but also to enhanced resistance to phagocytic clearance, as recently demonstrated in human whole blood killing assays (Courtney *et al.*, 2006a).

GAS strains have been defined as belonging to either class I or class II M types based on epitopes present within the conserved C repeat region of M proteins (Bessen *et al.*, 1989; Bessen and Fischetti, 1990) and the architecture of the *mga* regulon (Haanes *et al.*, 1992). The expression of SOF is found exclusively in Class II GAS (Bessen *et al.*, 1989). Given that SOF is expressed by only a subset of GAS strains (40-50%) the opacity factor is not a requirement for virulence in all GAS strains. However, this study found that specific deletion of SOF in the Class II M49 GAS background significantly reduced virulence in the mouse model of necrotizing skin infection. Different GAS strains express a wide array of surface anchored proteins that interact with components of the extracellular matrix (Ramachandran *et al.*, 2004), and a number of these have been shown to play a role in adherence of the organism to host epithelial cell surfaces and the invasion of the GAS bacterium into these cells (Caparon *et al.*, 1991; Courtney *et al.*, 1997; Hanski and Caparon, 1992; Jaffe *et al.*, 1996; Kreikemeyer *et al.*, 2004; Molinari *et al.*, 1997; Wang and Stinson, 1994). Differential expression of multiple adhesins in response to varying environmental cues and extracellular matrix components at specific tissue sites may be important for colonization of a tissue specific site. Thus, SOF may represent a critical virulence factor of Class II SOF(+) GAS, the absence of which in Class I GAS may be compensated for by the presence of other fibronectin-binding surface adhesins.

In summary, our studies demonstrate that SOF can contribute to epithelial cell invasion and animal virulence in the approximately 50% of clinical isolates that express this protein. Previous assumptions that interactions with the host cell were mediated predominantly through the C-terminal repeat domains should now be reconsidered, as both opacification and promotion of host cell invasion appear to be mediated by the SOF N-terminal domain.

EXPERIMENTAL PROCEDURES

Bacterial strains and culture conditions. Serotype M49 GAS strain NZ131 is a well-characterized T14 SOF(+) strain originally isolated from the skin of a patient with glomerulonephritis (Simon and Ferretti, 1991). Isogenic mutants NZ131ΔSOF and NZ131ΔSfbX contain a precise, in-frame allelic replacement of the corresponding open reading frame with a chloramphenicol acetylase (*cat*) gene marker (Jeng *et al.*, 2003). Serotype M1 GAS strain 5448 is a T1, SOF(-) isolate from a patient with necrotizing fasciitis and toxic shock syndrome (Kansal *et al.*, 2000). *Lactococcus lactis* strain NZ9000 is derived from MG1363 and lacks the *nis* operon (Kuipers *et al.*, 1998). SOF and SfbX amplified from NZ131 were cloned in vector pDCerm to yield plasmids pAJ-SOF and pAJ-SfbX (Jeng *et al.*, 2003), and used to transform M1 GAS 5448 and *L. lactis* for heterologous expression studies in comparison to vector only controls. GAS were grown in Todd-Hewitt broth (THB), on Todd-Hewitt agar plates (THA), or on plates of Trypticase Soy Agar + 5% sheep red blood cells (SBA). Antibiotic selection for plasmid maintenance utilized 2 μg/ml of erythromycin (Em) for GAS and 5 μg/ml

Em for *L. lactis*. To prepare log phase GAS for use in tissue culture assays or animal infections, bacteria were grown at 37°C to OD_{600} = 0.4 = 2 x 10^8 cfu/ml, pelleted, washed, resuspended and diluted in PBS or tissue culture media to the desired concentration. *L. lactis* was prepared as above (OD_{600} = 0.4 = 10^8 cfu/ml) but incubation performed at 30°C.

Serum opacification assays. Bacteria were grown to stationary phase overnight, pelleted by centrifugation, and 100 μl of supernatant added to 1 ml of heat inactivated (55°C, 30 min) horse serum (Gibco) in a 24-well cell culture plate. The plate was rocked for 15 min, sealed in a plastic bag with moisture, and incubated overnight at 37°C. Opacification was observed and photographed on a dark background.

Epithelial cell invasion assays. Human pharyngeal epithelial cells (HEp-2) were obtained from ATCC and propagated as monolayers in RPMI 1640 (Mediatech) supplemented with 10% FBS + 2 mM L-glutamine + 100 μM non-essential amino acids at 37°C with 5% CO_2. For assays, HEp-2 cells were plated at 2 x 10^5 cells/well in one mL of media in a tissue culture treated 24 well plate and incubated at 37°C x 24 h. Bacterial cultures were inoculated from a single colony and grown overnight in THB with corresponding antibiotic selection. The following day, cultures were diluted 1:10 in fresh THB without antibiotics and grown to an OD_{600nm} of 0.4 (~2 x 10^8 cfu/ml). Immediately prior to assay, the

culture media on the HEp-2 cells was replaced with fresh RPMI 1640 + 2% FBS. Bacteria were diluted in PBS and 10^6 cfu added to each well for a multiplicity of infection of 5:1 (bacteria:cell). The plate was centrifuged at 800 x g x 5 min to ensure contact of the bacteria with the cell monolayer, then incubated at 37°C in 5% CO_2. After 2 h, monolayers were washed x 3 with PBS to remove unattached bacteria. Media containing antibiotics (100 μg/ml gentamicin + 5 μg/ml penicillin) was added to each well, and the plates incubated an additional 2 h to kill extracellular bacteria. The wells were washed again, and 100 μl of trypsin added to each well for 3-5 minutes at 37°C to facilitate cell detachment. Triton-X 100 was added to each well at a final concentration of 0.025% and triturated with a micropipette for 20 sec to liberate intracellular bacteria. Dilutions were plated on THB at 37°C overnight for enumeration of cfu. All assays were carried out in triplicate and repeated 4-6 times.

For the M75 strain invasion assays, the HEp-2 cells were plated in 96 well plates at 4 x 10^4 cells/well in 200 μl media on the previous day. The purified SOFΔFn was preincubated with the cells for one hour prior to addition of the bacteria at an MOI of 5:1. The soluble protein was kept on during the first two hour incubation step of the assay, and the rest of the assay carried out as above.

Epithelial cell adherence assays. Assays were performed as described above, but after two hours of incubation, wells were washed x 6 with PBS to

remove unattached bacteria. Cells were then lysed, diluted, and plated as described.

Fluorescence microscopy. HEp-2 cells were prepared as described in the invasion assay protocol. Bacteria were grown to an OD_{600nm} of 0.3 (GAS) or 0.2 (*L. lactis*), then 1 ml pelleted by centrifugation at 10,000 x *g.* Bacteria were resuspended in 100 μl PBS + 10% THB, 0.5 μl 10 mM calcein-AM (Molecular Probes) added, and staining carried out for 1 h at 37°C (GAS) or 2 h at 30°C (*L. lactis*). Stained bacteria were washed x 3 with PBS to remove residual calcein-AM, resuspended in 1 ml PBS, 100 μl added to each HEp-2 monolayer, the plate centrifuged, and incubated for 2 h at 37°C to allow for internalization. After incubation, wells were washed with PBS, counterstained for 1-3 min with 100 μg/ml ethidium bromide, and washed again. Fluorescence was visualized with a Nikon TE200 inverted microscope using a green filter, a red filter, and white light. Images were captured with a CCD camera and assembled using Adobe Photoshop Creative Suite 2.

Construction and expression of pAT-SOFΔFn. This plasmid was generated by modification of pAJ-*sof* (Jeng *et al.*, 2003) through inverse PCR and blunt-end ligation. Primers were designed (fwd: 5'-ttcttccactttgataacgag-3', rev: 5'-ggcagtaatggattcaatgga-3') to create an inverse PCR product that consisted of the entire plasmid and gene, excluding the C-terminal fibronectin binding repeat region (residues 831Q to 960R). The resultant PCR product was

purified (Qiagen Gel Extraction Kit), blunt-end ligated for 2 h at RT using Invitrogen T4 ligase and used to transform *E. coli* MC1061 with selection for Em resistance. Resultant plasmid pAT-SOFΔFn was purified and confirmed by restriction analysis and direct sequencing. The pAT-SOFΔFn was used to transform the GAS M49 ΔSOF mutant and *L. lactis* by electroporation at 1700 volts, recovery in THB 0.25% sucrose, and Em selection. Transformants were confirmed by PCR analysis and gain of serum opacification phenotype.

Survey of GAS clinical strains for correlation between SOF phenotype and HEp-2 cell invasion. Single representatives of 45 different *emm* types were obtained from invasive isolates collected through the Centers for Disease Control Active Bacterial Core surveillance program in the United States during 2002-2004 (see http://www.cdc.gov/ncidod/dbmd/abcs/). These isolates were collected from patients of a wide age range (<1 to >90 years) and varied geographic locations, that represented a wide range of clinical manifestations. Strains were grown in 200 μl THB within individual wells of a 96-well plate with passage over 2 nights prior to use in the assay. HEp-2 cells were plated in a 96-well tissue culture treated plate at 4 x 10^4 cells/well in 200 μl of media and incubated x 24 h. On the day of the assay, 18 h stationary phase GAS cultures were serially diluted 1:100 in 96 well plates in RPMI 2% FBS, then 100 μl added to the HEp-2 cell monolayers in quadruplicate wells for each strain. The balance of the study was carried out as for the standard invasion assay, with serial dilutions plated on THB for enumeration of cfu.

Expression and purification of recombinant SOF75ΔFn protein. A His_6-tagged fusion protein of residues 33 to 872 of SOF from M75 GAS strain (lacking signal sequence and fibronectin-binding repeat region, SOF75ΔFn) was constructed using the pQE30 vector system (Kreikemeyer *et al.*, 1999). Large-scale expression and purification of SOF75ΔFn was conducted essentially according to manufacturer's instructions (Qiagen, Chatsworth, CA). Briefly, recombinant protein was expressed in *E. coli* M15 (pREP4)(pSOF75ΔFn), 1L culture in LB was grown to mid-log phase, and protein expression was induced by the addition of IPTG to a final concentration of 1 mM and incubation for a further 4 h at 37^{o}C with shaking. Cells were harvested by centrifugation at 4000 x *g* for 20 minutes at 4^{o}C and cell pellets stored at -20^{o}C overnight prior to protein extraction and purification. Large-scale purification of (His_6)-tagged SOF75ΔFn was performed using Ni-NTA chromatography under denaturing conditions according to manufacturer's instructions (Qiagen, Chatsworth, CA). To ensure slow buffer exchange for refolding of the denatured proteins, the proteins were initially dialyzed against 2 changes of 0.5-1 L PBS followed by 2 changes of 3 L PBS. To ensure correct refolding of proteins was achieved, purified proteins were subsequently tested for opacity factor activity using an agarose overlay method (Kreikemeyer *et al.*, 1999).

Interaction of SOF coated latex beads with HEp-2 cells. As per previously published methods (Dombek *et al.*, 1999; Molinari *et al.*, 1997), latex

beads (3 μm; Sigma-Aldrich, St. Louis, MO) were coated with purified recombinant SOF75ΔFn. Briefly, 10^8 bead particles in 50 μl of PBS were incubated with 5 μg of purified protein in PBS overnight at 4°C. After washing steps, free binding sites on the bead surface were blocked by incubation with 200 μl of 10 mg/ml BSA in PBS for 1 h at room temperature. Followed by washing and resuspension in a final volume of 1 ml Dulbecco modified Eagle medium (DMEM) (Invitrogen, Karlsruhe, Germany) with HEPES and 1% fetal calf serum (FCS). The efficiency of protein loading was verified by incubation of ~2 x 10^7 coated beads with polyclonal anti-SOF75 rabbit serum (diluted 1:1000 PBS + 5 mg/ml BSA) (Gillen *et al.*, 2002) followed by washing in PBS and incubation with goat anti-rabbit Alexa 488 (green) (50 μl of a 1:40 dilution in PBS + 5 mg/ml BSA) (Molecular Probes, Eugene, OR) for 1 h at room temperature and subsequently washed with PBS. Bead fluorescence was then visualized by fluorescent microscopy (Zeiss inverted microscope x 100 M), where 100% of observed beads were coated with protein (data not shown). HEp-2 cells were seeded on 12-mm-diameter glass coverslips (Nunc, Wiesbaden, Germany), placed on the bottom of 24-well tissue culture plates (Nunc, Wiesbaden, Germany) at 2 × 10^5 cells per well and allowed to grow in DMEM supplemented with 10% FCS (FCS; GIBCO), 5 mM glutamine (FlowLaboratories, Inc., McLean, Va.), penicillin (100 U/ml), and streptomycin (100 mg/ml) to semi-confluent monolayers at 37°C in a 5% CO_2 atmosphere. Cells were washed twice with DMEM HEPES, and 500 μl of DMEM HEPES + 1% FCS was added to the cells followed by the addition of

200 μl of the bead suspension and incubation for 2 h at 37°C in a 5% CO_2 atmosphere. Cells were washed vigorously with PBS to remove unbound beads and processed for scanning electron microscopy.

Electron microscopy studies. Cells were fixed in a solution containing 3% glutaraldehyde and 5% formaldehyde in cacodylate buffer for 45 min on ice and then washed with cacodylate buffer. For scanning electron microscopy, samples were dehydrated in a graded series of acetone and subjected to critical-point drying with CO_2. Samples were then covered with a 10-nm-thick gold film and examined with a Zeiss 982 Gemini digital scanning microscope.

Mouse infection models. For systemic challenges, 10-week old male CD-1 mice were infected intraperitoneally with the specified dose of GAS strains (2×10^7 cfu for WT GAS M49 and isogenic mutants, 2×10^6 cfu for WT M1 GAS and transformant expressing SOF49) in 5% mucin. Mortality was monitored and recorded for a 10-day period. An established model of GAS necrotizing subcutaneous infection was also used (Buchanan *et al.*, 2006; Datta *et al.*, 2005). Briefly, logarithmic phase GAS mutant were pelleted, resuspended in PBS, diluted 1:1 with sterile Cytodex beads (Sigma), and injected subcutaneously at the specified dose in 100 μl volume into shaved 10-week-old male CD-1 mice. WT SOF(+) GAS strain M49 was injected into one flank and its isogenic ΔSOF mutant simultaneously in the opposite flank for direct comparison in each mouse. The developing lesion sizes were measured daily. At the end of the experiment,

mice were sacrificed, the lesion removed by excisional biopsy, homogenized, and serially diluted in PBS for plating on THA and enumeration of cfu/lesion.

Statistical analyses: Data sets were compared by Student's T-test using the Microsoft Excel statistical package; a *P* value < 0.05 was considered significant.

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Chapter III

IL-8 Protease SpyCEP Contributes to Neutrophil Resistance and Virulence of Invasive Group A *Streptococcus*

ABSTRACT

Interleukin-8 (IL-8) plays a pivotal role in neutrophil-mediated host defense through its activities as a potent chemoattractant, activator of neutrophil bactericidal activities, and inhibitor of neutrophil apoptosis. Group A *Streptococcus* (GAS) protease SpyCEP (aka ScpC) cleaves IL-8. The encoding gene *cepA* is among the most strongly upregulated genes *in vivo* of clonal serotype M1T1 GAS strains associated with life-threatening systemic disease including necrotizing fasciitis (NF). Coupling allelic replacement mutagenesis with heterologous gene expression, we demonstrate that SpyCEP is necessary and sufficient for M1T1 GAS IL-8 degradation. The protease markedly decreases interleukin-8 dependent neutrophil endothelial transmigration and neutrophil killing by reduction of neutrophil extracellular traps (NETs) formation. SpyCEP also contributes to Group A *Streptococcus*-induced neutrophil apoptosis. In comparison to the WT parent M1T1 GAS strain, the isogenic Δc*epA* knockout mutant is attenuated for virulence in a murine model of NF. We conclude that through inactivation of the multifunctional host defense peptide IL-8, the SpyCEP protease impairs neutrophil clearance mechanisms and contributes to the pathogenesis of invasive M1T1 GAS 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 (Carapetis 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 described by the nine surveillance centers of the United States Centers for Disease Control Emerging Infections Program Network each year from 1997 though 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 vs. 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. The second most highly upregulated gene (25-fold) in the invasive GAS

transcriptional profile was that encoding SpyCEP (aka ScpC, PrtS), a predicted 1,645 amino acid protein containing a predicted C-terminal LP(X)TG cell wall anchor, 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 a 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). 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 in M1T1 GAS resistance to phagocytic clearance and animal virulence. To test this hypothesis, we generated

a single deletion mutant of *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 nonpathogenic 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 a murine model of NF.

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 5448Δ*cepA* (**Fig. 3.1**). The intact M1T1 *cepA* gene was also cloned in the *Escherichia coli*-streptococcal shuttle expression vector pDCerm (Jeng et al., 2003), generating p*cepA*, 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 p*cepA* plasmid (**Fig. 3.2**).

SDS-PAGE showed generation of the 6 kD SpyCEP IL-8 cleavage product after coincubation of the supernatants from the WT GAS parent strain,

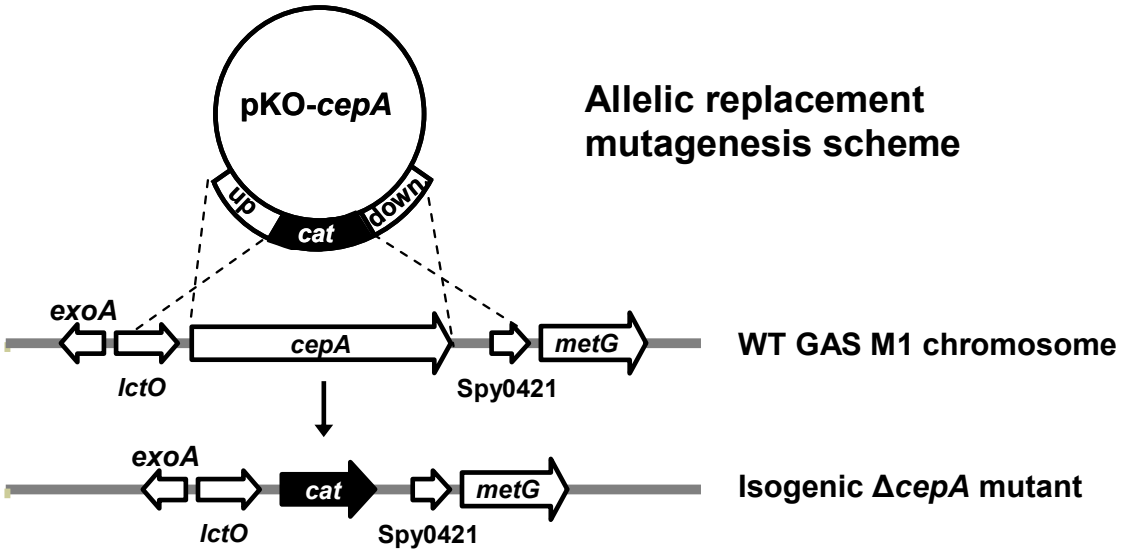

Figure 3.1 Scheme for precise, in-frame allelic replacement of the *cepA* gene in M1 GAS.

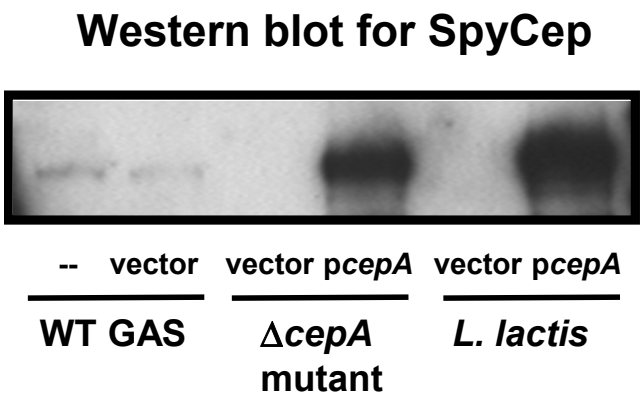

Figure 3.2 Western blot confirms loss of SpyCEP expression upon targeted mutagenesis in M1 GAS, complementation of the mutant *in trans*, and heterologous expression of SpyCEP in *Lactococcus lactis.*

complemented Δ*cepA* mutant, and *L. lactis* expressing p*cepA* with IL-8, but the cleavage product was absent in the Δ*cepA* mutant or *L. lactis* containing the empty vector control (**Fig. 3.3A**). Western blot analysis (**Fig. 3.3B**) and IL-8 ELISA (**Fig. 3.3C**) confirmed IL-8 protease activity by supernatants of the corresponding strains expressing SpyCEP and its absence in strains lacking the GAS 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.

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 Δ*cepA* mutant and untransformed *L. lactis* was significantly greater than observed with the WT GAS parent strain, complemented Δ*cepA* mutant or *L.*

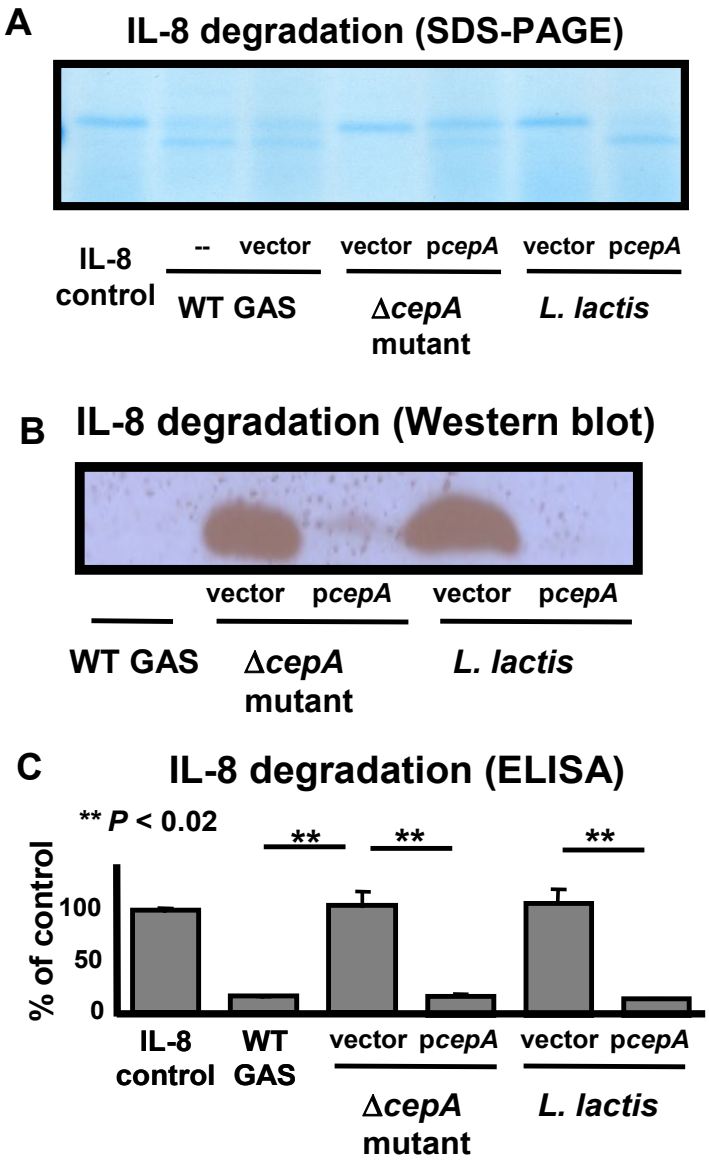

Figure 3.3 Presence of SpyCEP correlates with IL-8 protease activity by SDS-PAGE (a), anti-IL-8 Western blot (b), and ELISA (c).

lactis expressing p*cepA* (**Fig. 3.4**). 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 Δ*cepA* mutant bacteria ($P < 0.005$); WT levels of neutrophil resistance were restored by complementation of the mutant with the p*cepA* expression vector (**Fig. 3.5**). Heterologous expression of SpyCEP in *L. lactis* increased survival of that bacterium in the neutrophil killing assay by ~50% ($P < 0.005$) (**Fig. 3.5**). To better understand the increased killing of the Δ*cepA* mutant bacteria, we looked at the effect SpyCEP may have on the rate of neutrophil apoptosis and the production of neutrophil extracellular traps.

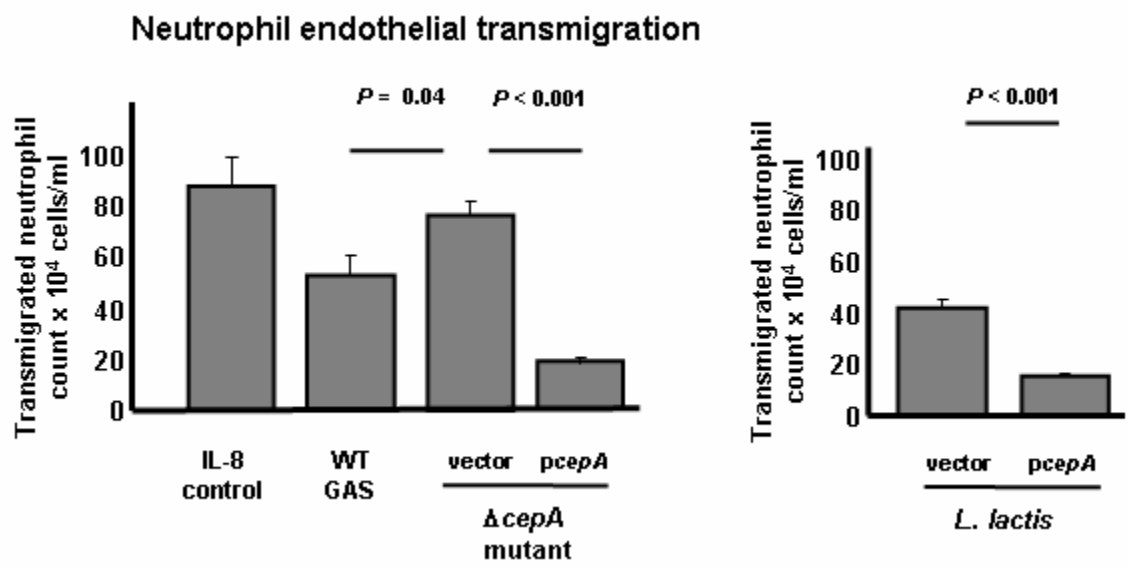

Figure 3.4 SpyCEP degradation of IL-8 impairs human neutrophil transmigration across an endothelial monolayer. Recombinant IL-8 was used as a positive control.

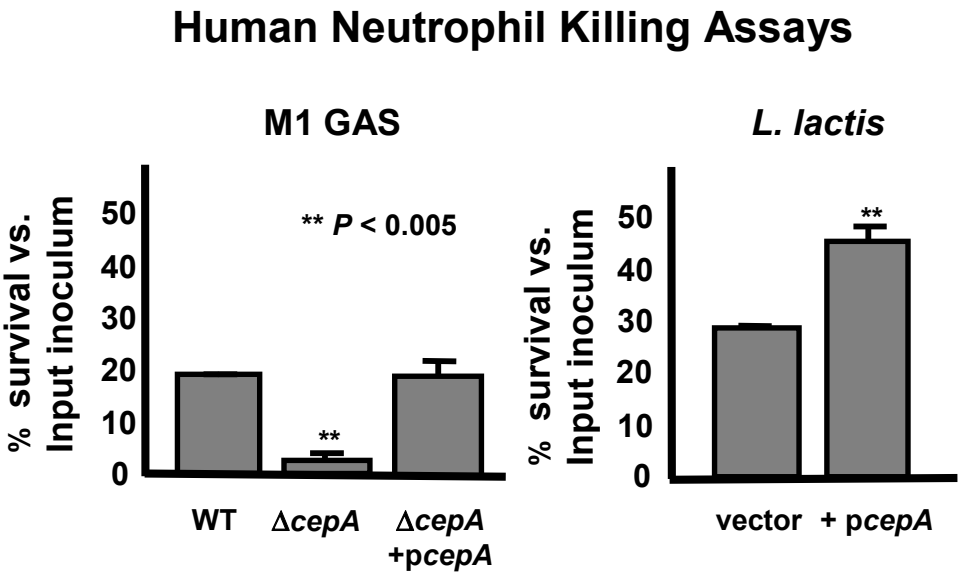

Figure 3.5 SpyCEP contributes to GAS survival and increases *Lactococcus lactis* survival upon co-incubation with human neutrophils.

SpyCEP promotes neutrophil apoptosis. GAS are known to promote a program of accelerated apoptosis in human neutrophils, which may contribute to pathogen survival and disease (Kobayashi et al., 2003; Miyoshi-Akiyama et al., 2005); however, the specific virulence factors responsible for this phenotype are as yet unknown. Tumor necrosis factor-α (TNF-α) is known to stimulate a late survival effect in neutrophils (Dunican et al., 2000), and this has recently been demonstrated to involve nuclear factor-κB and ERK dependent production of IL-8, which acts in turn to suppress neutrophil apoptosis in a paracrine manner (Cowburn et al., 2004; Grutkoski et al., 2002). We hypothesized that SpyCEP-mediated degradation of IL-8 could eliminate this anti-apoptotic pathway, contributing to the observed GAS capacity to induce neutrophil apoptosis. As shown in **Fig. 3.6**, deletion of *cepA* resulted in a significant decrease in the ability of GAS to induce neutrophil apoptosis.

SpyCEP reduces neutrophil production of extracellular traps (NETs). 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). NETs are induced by exposing human neutrophils to IL-8 (Brinkmann et al., 2004; Gupta et al., 2005). We thus

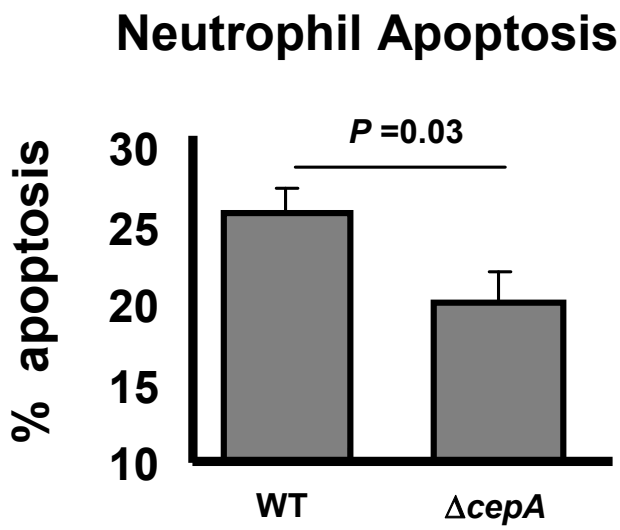

Figure 3.6 SpyCEP contributes to GAS-induced neutrophil apoptosis as measured by TUNEL assay.

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 (**Fig. 3.7**). 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 **Fig. 3.7**, NETs were significantly more abundant in human neutrophils incubated with the Δ*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 (**Fig. 3.7**). These data suggest that the IL-8 degrading activity of SpyCEP can impair NET production as an additional contributor to neutrophil resistance.

SpyCEP impedes GAS epithelial cell adherence and invasion. One factor that may contribute to the virulence of the globally-disseminated M1T1 GAS clone is a capacity for high frequency intracellular invasion of epithelial cells (Cleary et al., 1998). Amino acid alignment shows that GAS *cepA* shares ~30% sequence identity and ~50% sequence similarity with the GAS C5a peptidase, ScpA. Recently, the ability of an M1 serotype strain of GAS to invade epithelial cells was shown to be inhibited both by antisera against ScpA and by exogenous addition of purified recombinant ScpA protein (Purushothaman et al., 2004),

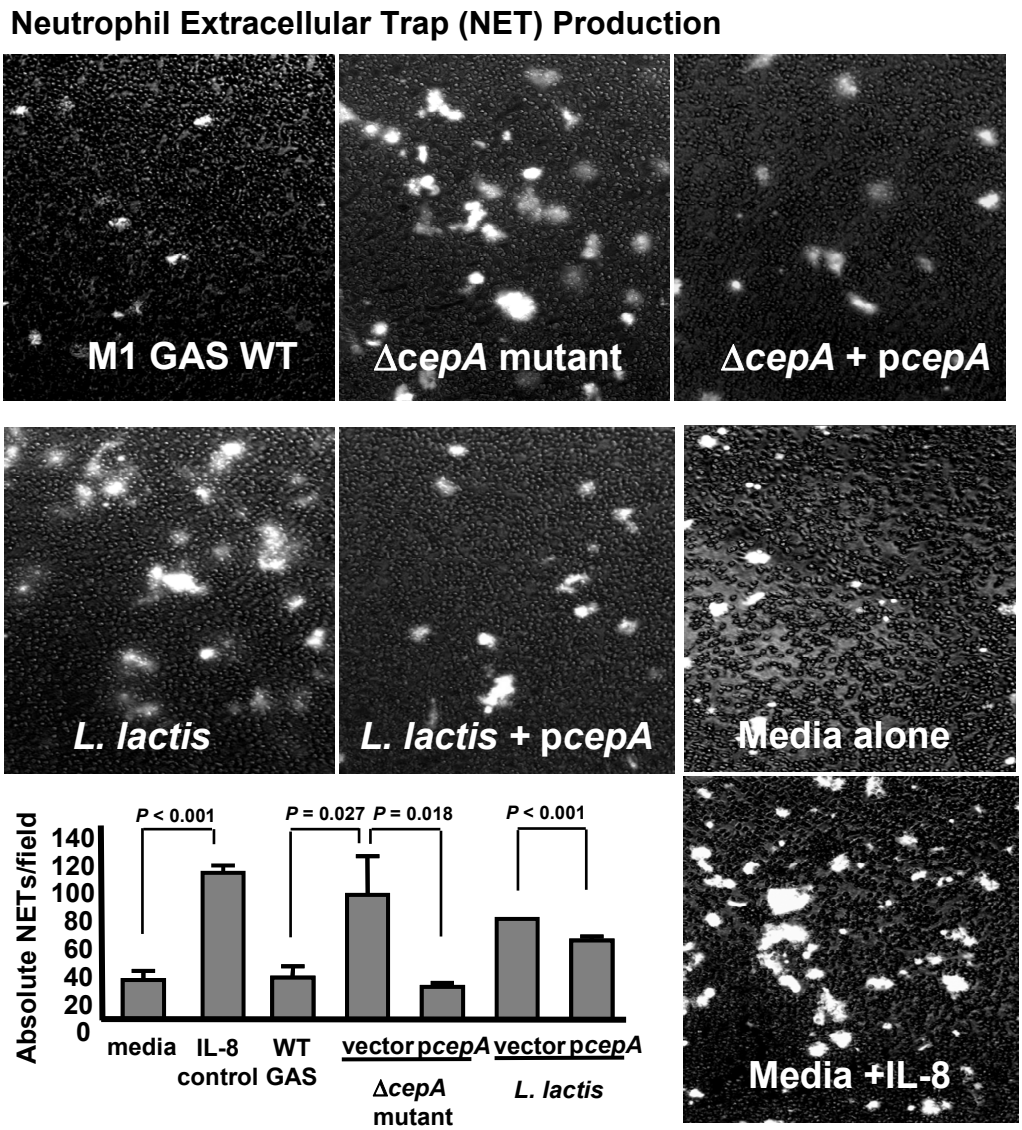

Figure 3.7 The presence of SpyCEP reduces induction of neutrophil extracellular traps (NETs) in response to bacterial exposure; fluorescent images of NETs stained with Sytox Orange and overlaid upon brightfield image of neutrophils; quantitative enumeration of NETs performed by direct visualization.

indirectly suggesting this surface anchored protease may function as a GAS epithelial cell invasin. We examined the contribution of SpyCEP to GAS adherence to and invasion of cultured HEp-2 human pharyngeal epithelial cells. The isogenic Δ*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 p*cepA* expression plasmid reversed the adherence and invasion phenotype to nearly WT levels (**Fig. 3.8**). These results show that in contrast to ScpA, SpyCEP does not act as a GAS epithelial cell adhesin or invasin.

SpyCEP contributes to M1T1 GAS virulence in a murine NF model. A murine subcutaneous challenge model can been used to examine the contribution of specific bacterial phentoypes to the virulence of M1T1 GAS *in vivo* (Buchanan et al., 2006; Datta et al., 2005; Sumby et al., 2005). Using C57B/6 mice, we injected one shaved flank with 5 x 10^7 cfu of the WT M1T1 GAS parent strain and the contralateral flank with an equivalent inoculum of the isogenic Δ*cepA* mutant. As shown in **Fig. 3.9**, the mean lesion size generated by the Δ*cepA* mutant was ~50% that of the WT lesions ($P < 0.02$). Representative histopathologic analysis showed increased neutrophil recruitment in lesions generated by the Δ*cepA* mutant compared to the WT parent strain (**Fig. 3.10**), confirming earlier indications that SpyCEP can impair murine neutrophil recruitment *in vivo* (Hidalgo-Grass et al., 2006).

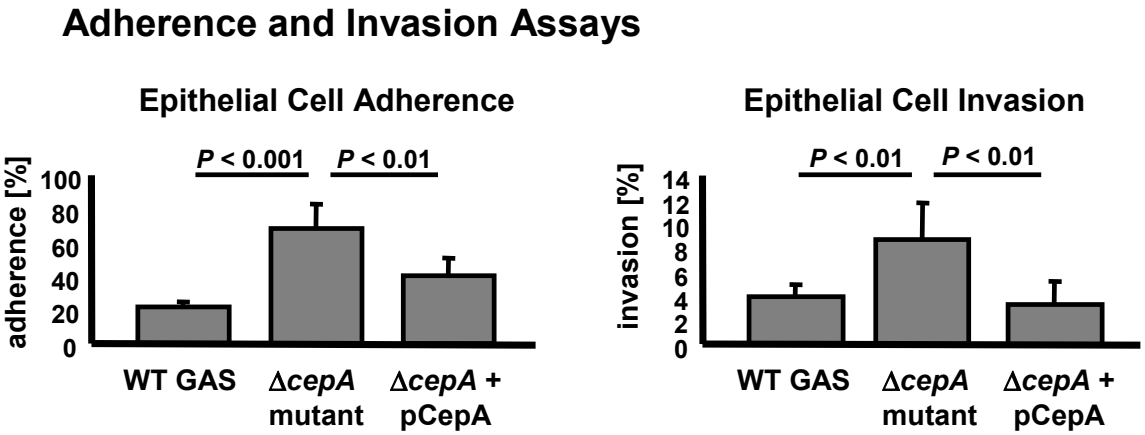

Figure 3.8 Elimination of SpyCEP increases the adherence and invasion of HEp-2 human pharyngeal epithelial cells by M1 GAS.

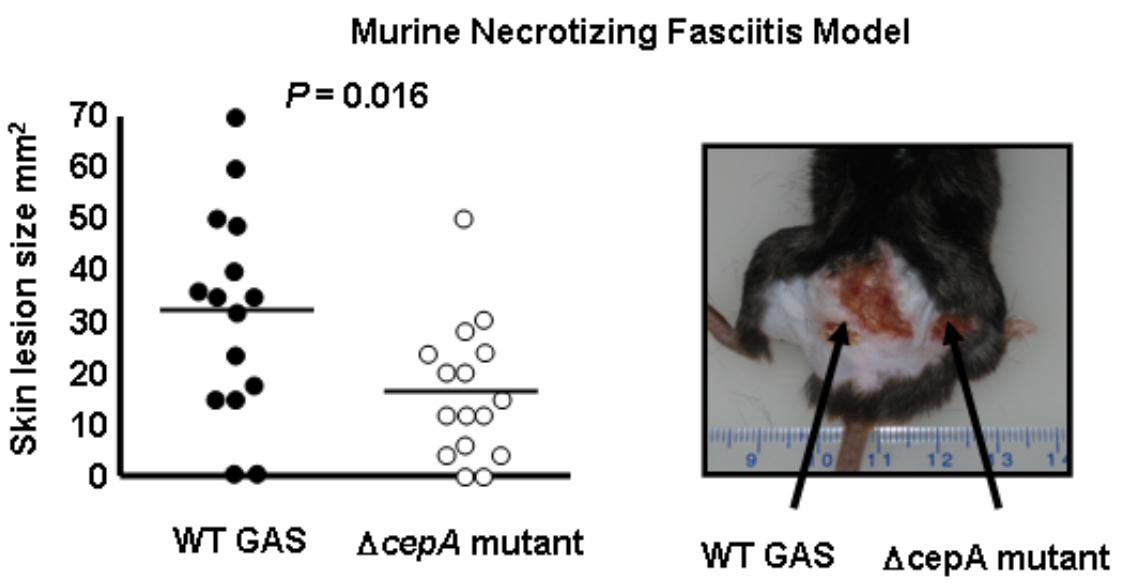

Figure 3.9 SpyCEP contributes to GAS virulence in a mouse necrotizing skin infection model. C57B/6 mice were injected subcutaneously with equivalent inocula of the WT M1 GAS strain (left flank) and the isogenic Δ*cepA* mutant (right flank) and skin lesion progression measured at 4 days. Representative photograph of skin lesions seen in subcutaneous challenge experiment.

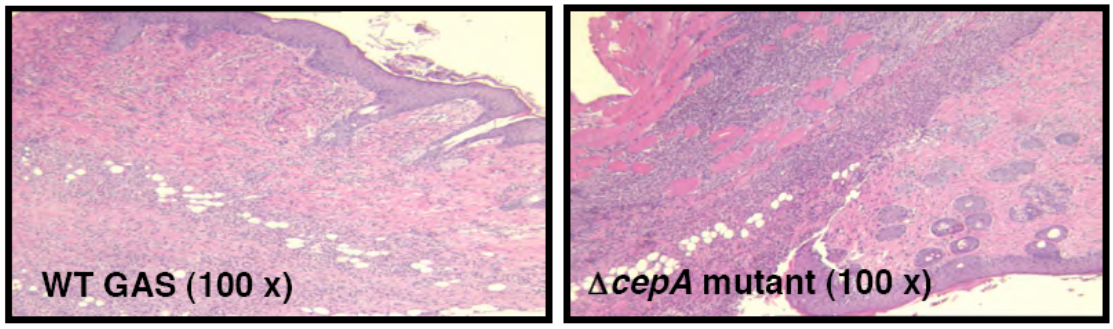

Figure 3.10 Representative histology (H&E stain) biopsies from skin lesions showing increased neutrophil recruitment at the Δ*cepA* mutant infection site (100 x magnification).

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 neutrophil resistance and virulence of M1T1 GAS. M1T1 had the anticipated effect of delaying neutrophil endothelial transmigration and recruitment to the tissues (Edwards et al., 2005; Hidalgo-Grass et al., 2006), and indeed a striking paucity of neutrophils at the site of infection 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, GROα) 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 and after dissemination into the bloodstream. 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. By removing the anti-apoptotic function of IL-8, SpyCEP can reduce neutrophil lifespan, identifying the protease as a specific molecular contributor to the accelerated apoptotic program induced by invasive M1T1 GAS (Kobayashi et al., 2003). The cumulative effect of these activities is consistent with our observation that expression of SpyCEP was necessary and sufficient to promote bacterial survival in killing assays with isolated neutrophils (**Fig. 3.11**).

The gene encoding SpyCEP was one of the most highly differentially expressed upon comparison of the transcriptional profile of invasive M1T1 GAS isolates vs. similar clonal strains isolated from the pharyngeal mucosa (Sumby et al., 2006). The observed 25-fold upregulation of *cepA* expression in the invasive

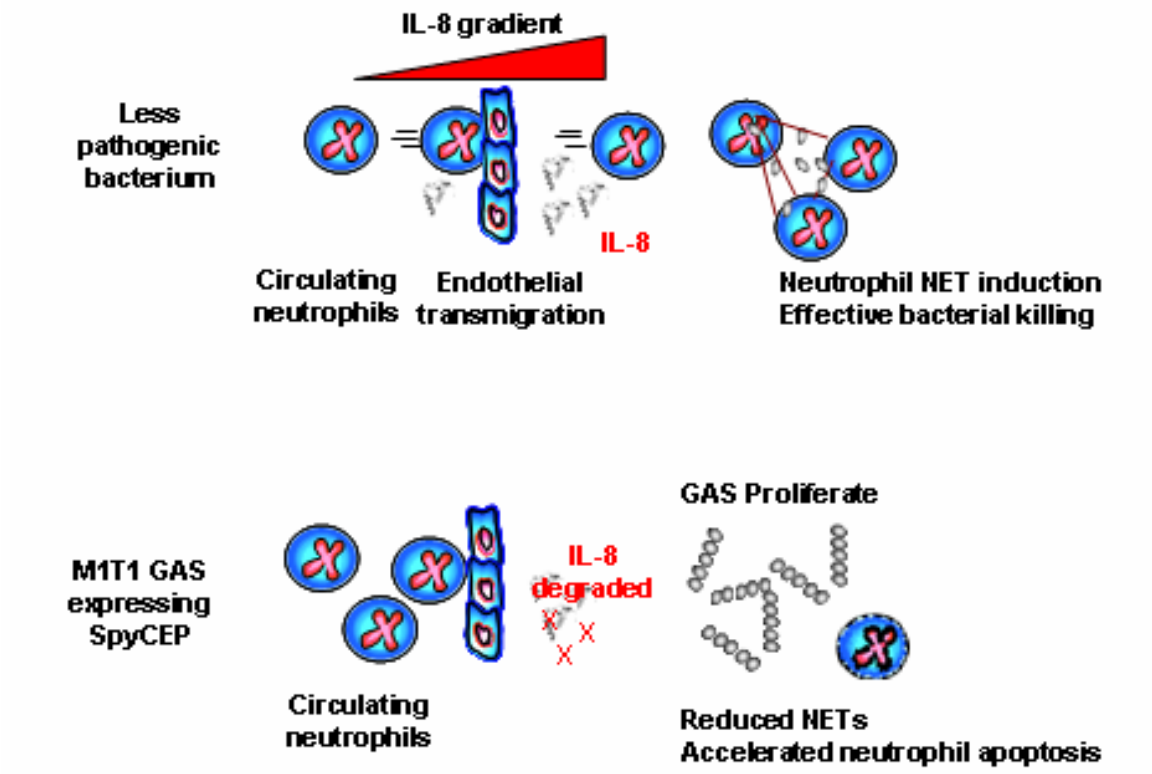

Figure 3.11 Schematic model depicting several mechanisms by which IL-8 protease SpyCEP can contribute to M1 GAS pathogenesis, including impairment of neutrophil chemotaxis, resistance to neutrophil bactericidal action, reduced induction of NETs, and accelerated neutrophil apoptosis.

transcriptional profile is not only consistent with our findings linking SpyCEP expression to M1T1 GAS resistance to neutrophil resistance, it suggests that high level expression of SpyCEP may be disadvantageous to the organism in the context of pharyngeal colonization. Our observation that SpyCEP interferes with GAS adherence to and entry within cultured human pharyngeal epithelial cells (**Fig. 3.8**) may provide a clue to the selective pressures driving differential regulation.

In sum, SpyCEP degradation of IL-8 compromises host neutrophil defense in multiple fashions and contributes to the pathogenesis of invasive M1T1 GAS infection (**Fig. 3.11**). Strategies directed at neutralizing SpyCEP activity could be 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). Nonpathogenic *L. lactis* strain NZ9000 has been used successfully for heterologous expression of GAS genes including the streptolysin S operon (Nizet et al., 2000), serum opacity factor (Jeng et al., 2003), and DNAse Sda1 (Buchanan et al., 2006). GAS and *L. lactis* were grown in Todd-Hewitt broth (THB) (Difco, BD Diagnostics) or TH agar plates, using 1 mg/ml chloramphenicol (Cm) or 2 mg/ml erythromycin (Em)

selection where appropriate. E. coli TOP10 (Invitrogen) were grown in Luria-Bertani (LB) broth or LB agar plates using 100 mg/ml ampicillin, 500 mg/ml Em or 5 mg/ml Cm selection. GAS and L. lactis were made competent for transformation by electroporation by growth in THB + 0.6% glycine. For use in neutrophil, epithelial cell, and mouse challenge studies, bacteria were grown to logarithmic phase in THB (OD600 =0.4 = ~ 2 x 10^8 cfu/ml), pelleted, washed and resuspended in PBS or tissue culture media at the desired concentration.

Allelic exchange mutagenesis. 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 (a) *cepA*-upF (5'-ggtataagacggggtcaaca-3', 526 bp upstream of the *cepA* start codon) with *cepA*-upR (5'-ggtggtatatccagtgatttttttctccatctgataccctcctaaatgtt-3'; immediately upstream of the *cepA* start site + 30 bp extension corresponding to 5' end of the *cat* gene) and (b) *cepA*-downF (5'-tactgcgatgagtggcagggcggggcgtaataacaaagcgcaaagagaca-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 was T-A cloned into Gateway 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. Precise, in-frame allelic exchange of *cepA* with *cat* in the GAS 5448Δ*cepA* mutant was confirmed by PCR analysis.

Complementation studies and heterologous expression of cepA*.* The M1 GAS *cepA* gene and flanking region 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.

Western blot and ELISA analyses*.* For IL-8 detection, bacteria were grown to mid-logarithmic phase, washed with PBS, and resuspended in RPMI + 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 h 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 Chemiluminiscent System (Pierce). For detection of SpyCEP protein by GAS and *L. lactis* strains, rabbit polyclonal antibody raised against a recombinant protein representing residues 35 to 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 co-incubated with an equal volume of human IL-8 (final concentration 5 μg/mL) at 37^{o}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 Transwell™ plates (6.5 mm diameter, 3 mm pore size, Biocoat, BD Biosciences). Human neutrophils were purified from healthy donors using the

PolymorphPrep™ system (Axis-Shield, Fresnius) and 1 x 10^6 cells added to the upper well of the chamber. Mid-log phase GAS or *L. lactis* at 1 x 10^7 cfu/ml were pre-incubated with 70 ng/ml of recombinant human IL-8 for 2 h at 37^{o}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^{o}C in 5% CO_2 before direct enumeration of neutrophils transcytosing to the lower well in a counter chamber (average of 4 high power fields).

Neutrophil 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^{o}C, added to neutrophils at final multiplicity of infection (MOI) = 2.5 bacteria/neutrophil, then incubated for 1 h at 37^{o}C with orbital rotation. 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. For quantification NETs were enumerated by counting 3 transects of 3 independent wells after staining with Sytox orange. Neutrophil apoptosis was measured using the APO-BrdU™ TUNEL Assay Kit (Molecular

Probes, Invitrogen) per manufacturer's instructions. Logarithmic phase GAS were added to purified neutrophils at MOI = 10 for 4 h and samples were analyzed by flow cytometry (FACS Calibur, Becton-Dickinson).

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 h 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-Alderich). At the end of the assay, wells were treated for 10 min with the fish antimicrobial peptide moronecidin (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 model*:* GAS virulence was tested using an established murine model of necrotizing skin infection (Buchanan et al., 2006; Nizet et al., 2001). Briefly, logarithmic phase GAS were resuspended in PBS, mixed 1:1 with sterile Cytodex beads (Sigma, St. Louis, MO), An inoculum of 5 x 10^7 cfu of WT

GAS was injected subcutaneously into one flank of 10-12 week old C57B/6 mice (Charles River Laboratories, CA), and simultaneously an equivalent inoculum of the isogenic Δ*cepA* GAS mutant was injected into the contralateral flank, allowing each mouse (N = 16) to serve as its own control. The size of developing necrotic lesions was monitored daily for four 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.

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Chapter IV

Group A *Streptococcus* Immune Evasion By Cytolysin-Mediated Accelerated Macrophage Apoptosis

ABSTRACT

Group A *Streptococcus* (GAS) is responsible for millions of human infections each year worldwide, including invasive conditions such as necrotizing fasciitis and septicemia. As frontline components of the host innate immune system, macrophages play a key role in the control and clearance of GAS infections. We find GAS induce rapid, dose-dependent apoptosis of primary and cultured murine macrophages. The cell death pathway, which was shown to be caspase-dependent and diminished in macrophages derived from caspase-1 knockout mice, required that GAS were internalized by the phagocyte. From a panel of virulence factor mutants, streptolysin O (SLO) was identified to play a major role in GAS-induced macrophage apoptosis; heterologous expression of SLO in the nonpathogenic *Lactococcus lactis* and administration of purified SLO demonstrated this pore-forming cytolysin to be sufficient to confer the macrophage apoptosis-inducing phenotype. SLO-deficient GAS mutants induced less macrophage apoptosis, greater macrophage cytokine secretion, and were less virulent in a murine systemic infection model. Ultrastructural evidence of mitochondrial membrane remodeling, coupled with loss of mitochondrial depolarization and cytochrome *c* release, suggest a direct attack of the toxin initiates the intrinsic pathway of apoptosis. A pan-caspase inhibitor blocked SLO-induced apoptosis and enhanced the killing of GAS upon co-incubation with macrophages. We conclude accelerated, caspase-dependent macrophage apoptosis induced by the pore-forming cytolysin SLO contributes to GAS immune evasion and virulence.

INTRODUCTION

Group A *Streptococcus* (GAS) is a leading human pathogen that annually infects hundreds of millions of people worldwide (Carapetis *et al.*, 2005). The last three decades have witnessed a marked increase in severe, invasive forms of GAS infection, many attributable to a single globally-disseminated clone of the M1T1 serotype (Cunningham, 2000). Invasive GAS infection defines a capacity of the pathogen to resist host innate defense mechanisms designed to prevent microbial spread beyond epithelial surfaces.

Macrophages are critical host defense cells involved directly in bacterial clearance and also in alerting other immune system components to invading pathogens. Macrophage microbicidal activity is accomplished by phagocytic uptake coupled with the action of reactive oxygen species, enzymatic proteolysis, and cationic antimicrobial peptides; their role in amplification of the innate and adaptive immune responses is achieved through release of soluble factors such as cytokines and nitric oxide. Mice depleted of macrophages or treated with inhibitors of macrophage phagocytosis are unable to clear GAS infections even following relatively low challenge doses (Goldmann *et al.*, 2004), demonstrating the essential first line defense function of these immune cells against the pathogen.

We sought to explore the interaction of the highly virulent GAS M1T1 clone with macrophages in order to better understand its propensity to produce

invasive human infection. A prominent regulatory feature of macrophage biology in the context of infectious disease and inflammation is the process of programmed cell death or apoptosis, mediated by member of the caspase family of cysteine proteases. While a number of highly adapted intracellular bacterial pathogens including *Mycobacterium tuberculosis*, *Legionella pneumophila*, and *Brucella* spp. have evolved mechanisms to block macrophage apoptosis and use the host cell as a vehicle for *in vivo* dissemination (Banga *et al.*, 2007; He *et al.*, 2006; Loeuillet *et al.*, 2006), a recent study of GAS M1T1 interactions with another host phagocytic cell type suggested a different outcome. In contrast to other prominent Gram-positive pathogens including *Staphylococcus aureus* and *Listeria monocytogenes*, GAS induced an accelerated program of apoptosis in human neutrophils (Kobayashi *et al.*, 2003); although the specific virulence factor(s) involved, effects on caspase activation, and contribution to disease outcome were not studied.

Here we report that GAS rapidly induce macrophage apoptosis through caspase-dependent pathways, initiated by release of cytochrome *c* by permeabilization of mitochondrial outer membranes. GAS-induced macrophage apoptosis is mediated by the cytolysin streptolysin O (SLO), which is both necessary and sufficient for the phenotype. SLO-mediated macrophage apoptosis leads to enhanced GAS survival, dampened cytokine responses, and increased virulence during systemic infection.

RESULTS

GAS induce rapid dose-dependent apoptosis of macrophages. To assess the ability of GAS to trigger macrophage apoptosis, murine macrophage cells (J774) were infected with GAS at various multiplicities of infection (MOI) for 1 h before addition of antibiotics to kill extracellular bacteria. At 4 h, macrophages were collected, fixed, permeabilized, and analyzed by terminal dUTP nick-end labeling (TUNEL) staining to quantify DNA fragmentation, a hallmark of apoptosis. GAS induced significant macrophage apoptosis in an inoculum-dependent fashion (**Fig. 4.1**). The kinetics of GAS-induced macrophage apoptosis (60% by 4 h at MOI = 10) were greatly accelerated compared to control treatments with RNA synthesis inhibitor actinomycin D (significant macrophage apoptosis seen beginning at 12-24 h) (**Fig. 4.2**). Macrophage apoptosis was not induced by penicillin-killed GAS nor by the nonpathogenic Gram-positive bacterium, *Lactococcus lactis* (LL) (**Fig. 4.3**), suggesting an association to a specific factor elaborated by living GAS. Inhibition of phagocytosis by an actin cytoskeleton inhibitor, cytochalasin D, markedly reduced GAS-triggered macrophage apoptosis (**Fig. 4.4**), indicating that GAS must be internalized to exert their proapoptotic effect(s).

GAS-induced macrophage apoptosis involves caspase activation. During apoptosis, stimulation of upstream, or initiator, caspases through multiple pathways ultimately converges upon activation of effector caspases responsible

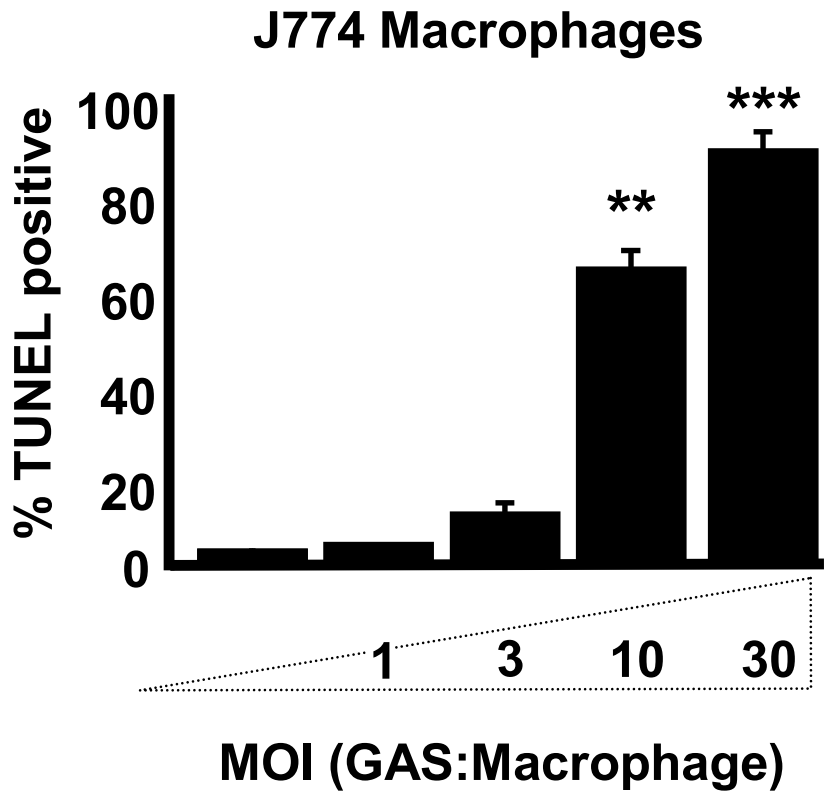

Figure 4.1 Dose-dependent induction of macrophage apoptosis by GAS (t = 4 h).

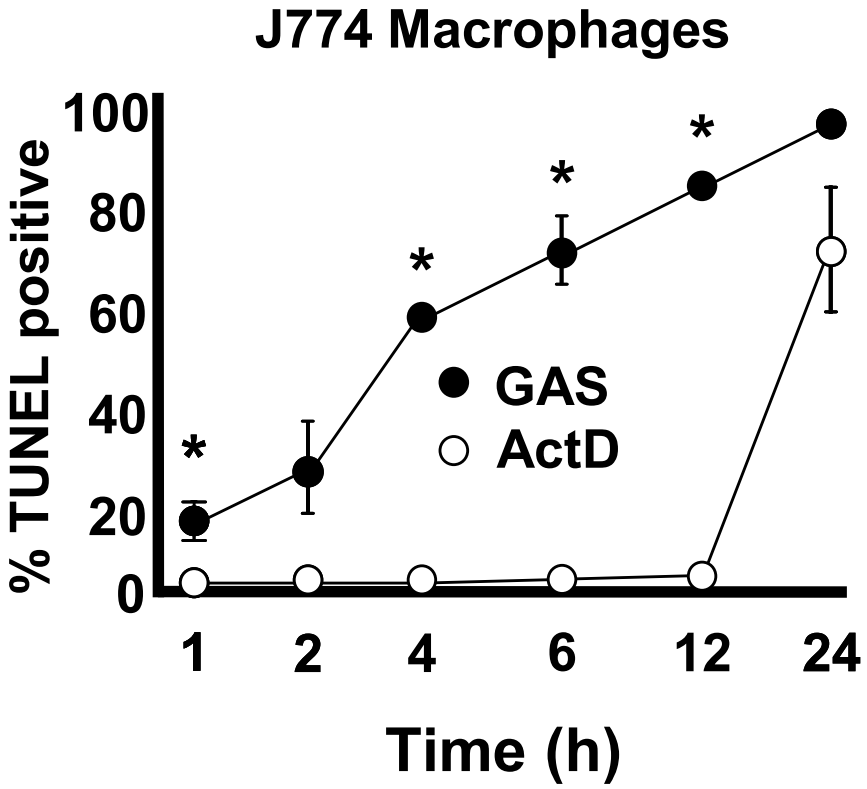

Figure 4.2 GAS induction of apoptosis proceeds more rapidly than control induction by actinomycin D, multiplicity of infection (MOI) = 10 bacteria/cell.

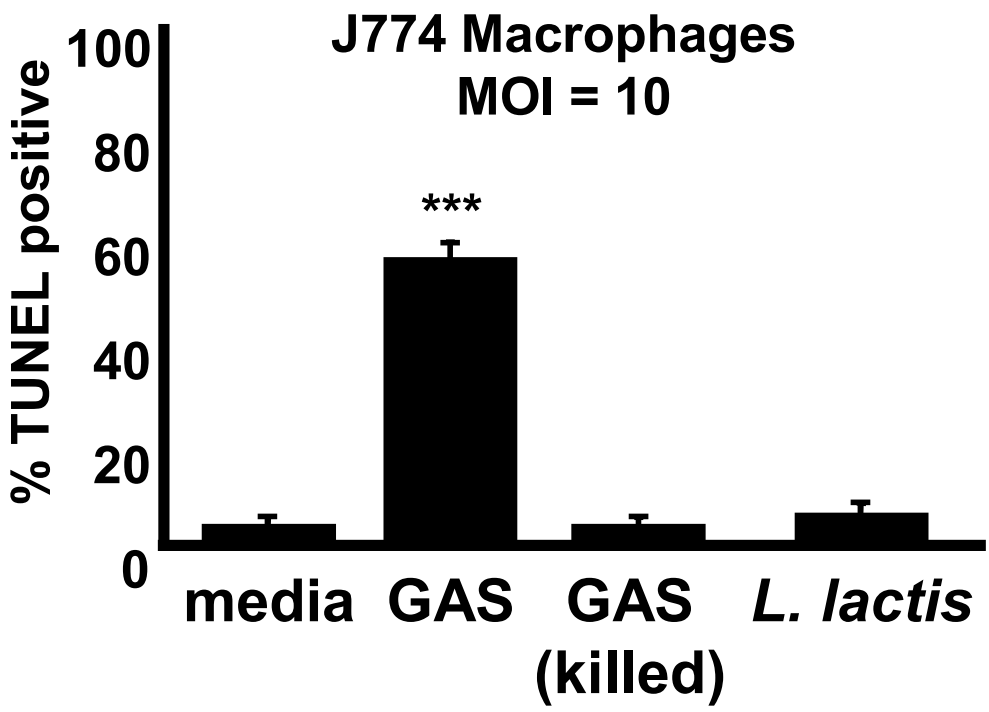

Figure 4.3 GAS must be viable to induce macrophage apoptosis.

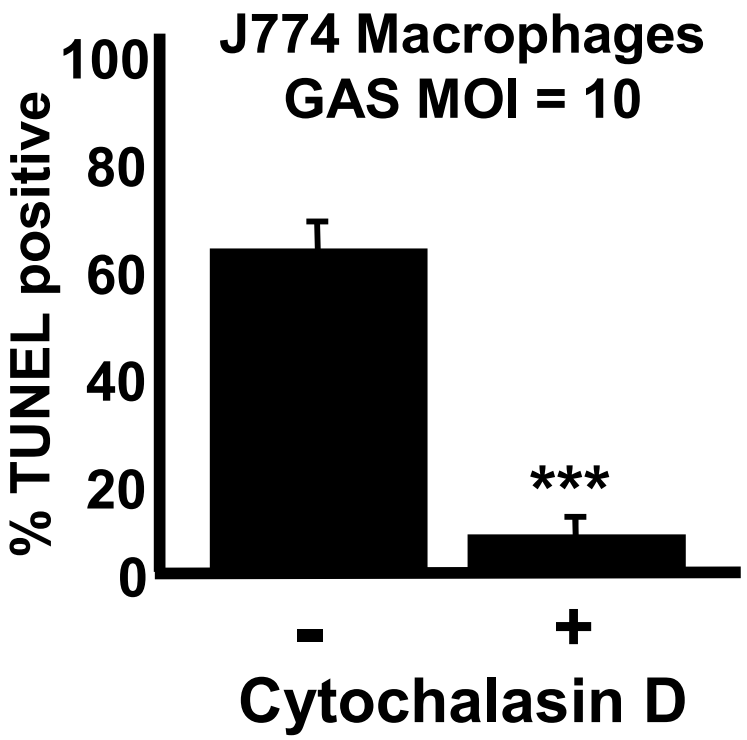

Figure 4.4 Internalization (phagocytosis) of GAS by the macrophage is required for apoptosis induction.

for organelle degradation and cell death. Pretreatment of J774 cells with pan-caspase inhibitor Q-VD-OPH significantly reduced apoptosis induced by GAS infection (**Fig. 4.5**). Using fluorescently-labeled peptide substrates, we found increased activity of the effector caspases-3/7 in lysates from macrophages infected with GAS but not in macrophages infected with LL or media control (**Fig. 4.6**).

Inflammatory caspase-1 has been implicated in programmed cell death during infections (Bergsbaken and Cookson, 2007; Hilbi *et al.*, 1998; Monack *et al.*, 2001; Sarkar *et al.*, 2006); sometimes the net effect of such activation is proinflammatory, leading to coinage of the term pyroptosis (Fink and Cookson, 2005). Caspase-1 activation occurs through the inflammasome, a complex of intracellular proteins formed upon detection of danger signals such as microbial factors or low intracellular potassium levels (Creagh and O'Neill, 2006). To test if caspase-1 activation contributed to GAS-induced macrophage apoptosis, we repeated assays in the presence of 130 mM KCl, which is known to inhibit inflammasome activation (Petrilli *et al.*, 2007). Elevated KCl significantly inhibited GAS-induced, but not actinomycin D-induced, macrophage apoptosis (**Fig. 4.7**). Pretreatment of macrophages with caspase-1-specific peptide inhibitor YVAD-CHO also led to a partial but significant decrease in apoptosis upon GAS infection (**Fig. 4.8**). Finally, GAS induced significantly less apoptosis in bone-marrow derived (BMD) macrophages from caspase-$1^{-/-}$ mice than in macrophages from wild-type (WT) control mice (**Fig. 4.9**). In sum these data

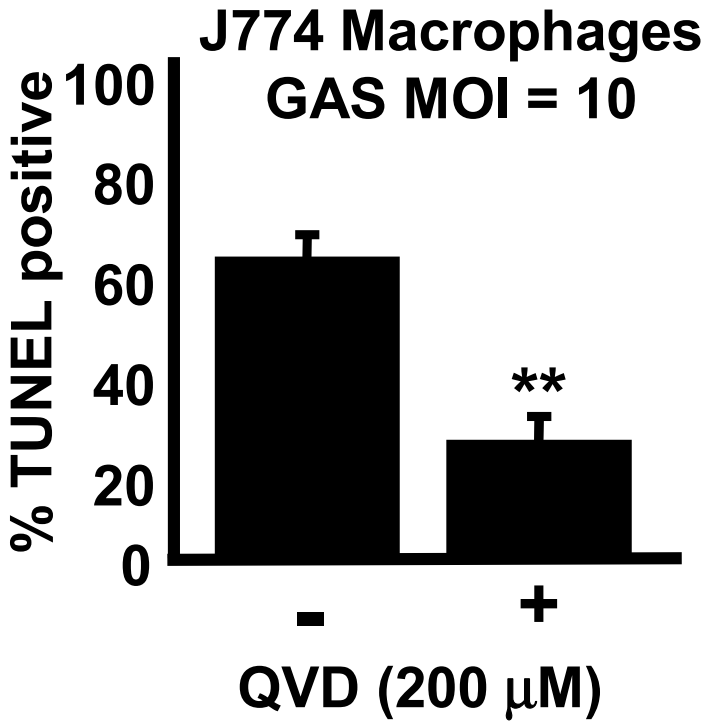

Figure 4.5 Induction of macrophage apoptosis is inhibited by pan-caspase inhibitor Q-VD-OPH.

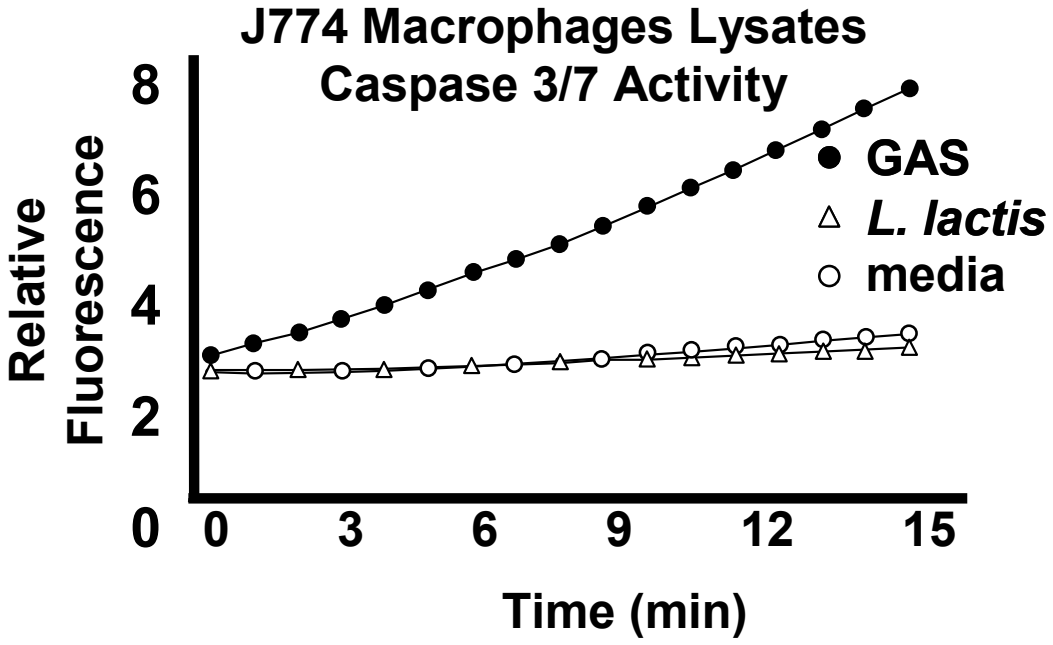

Figure 4.6 Cleavage of effector caspase-3/7 fluorescently-conjugated peptide substrates in lysates of cells infected with GAS but not *L. lactis* control.

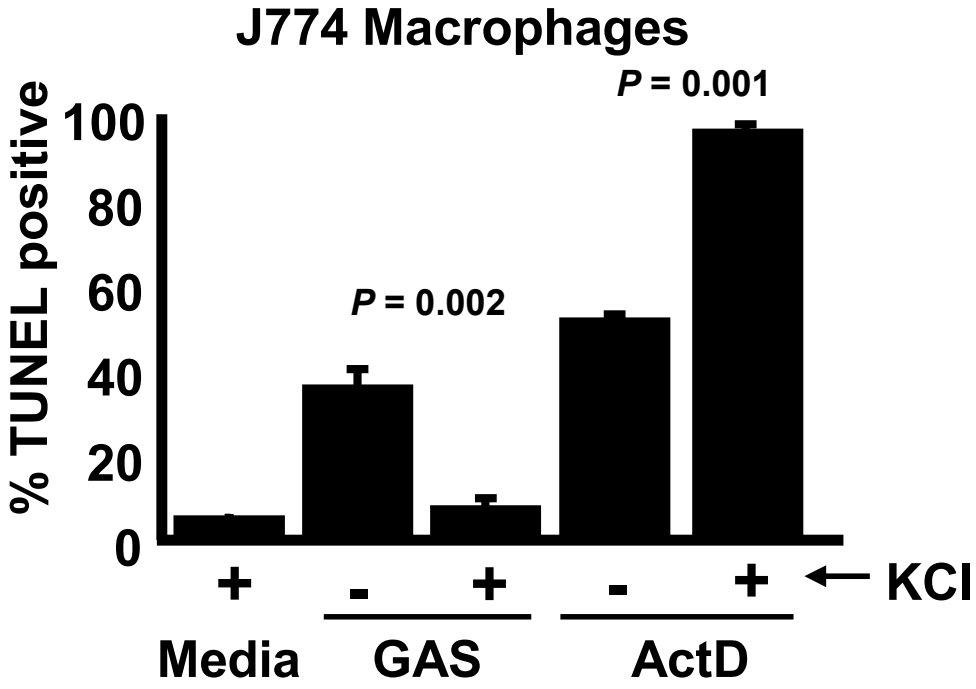

Figure 4.7 High extracellular KCl (130 mM) inhibits GAS-induced macrophage apoptosis.

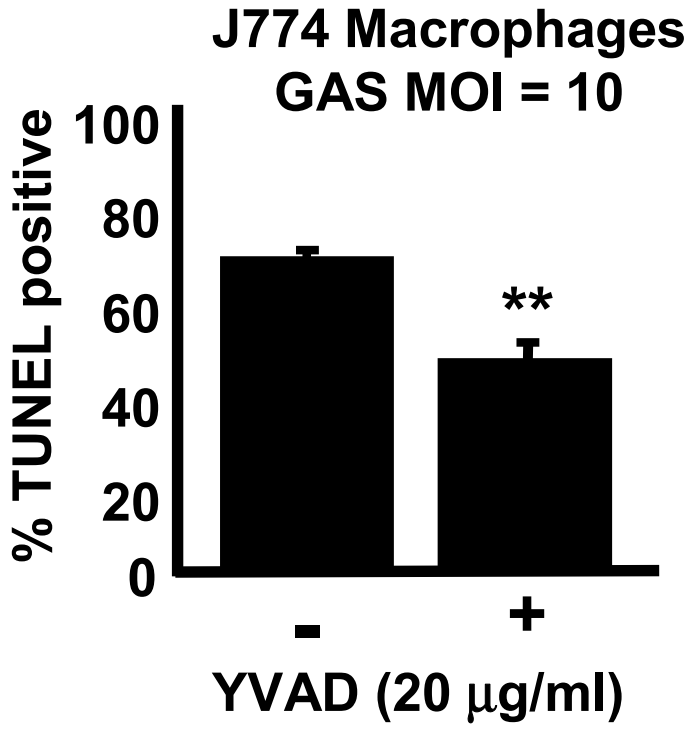

Figure 4.8 Caspase-1-specific inhibitor YVAD-CHO decreases GAS-induced macrophage apoptosis.

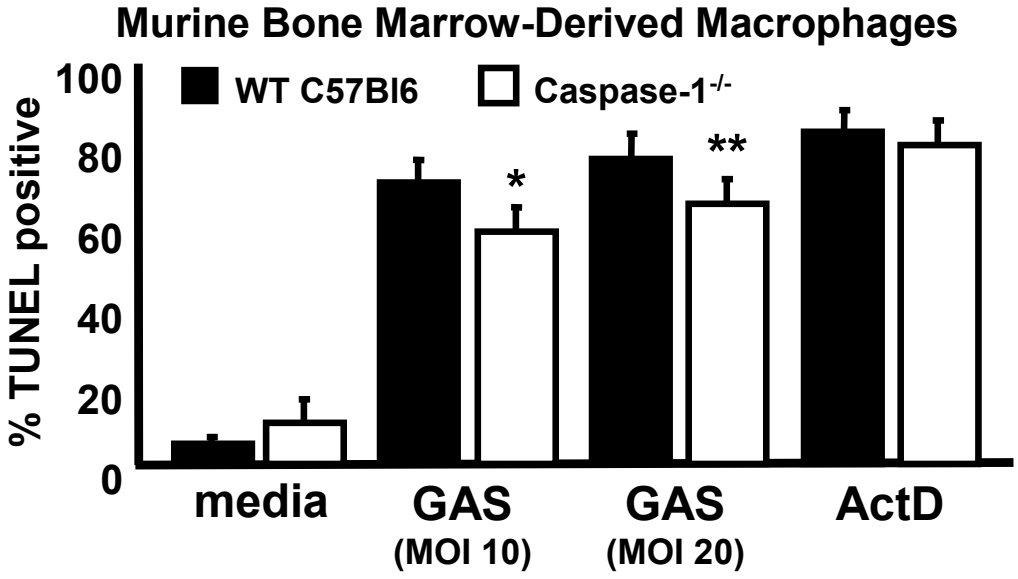

Figure 4.9 Caspase-$1^{-/-}$ mouse macrophages are more resistant to GAS-induced apoptosis.

demonstrate a caspase-dependent pathway of apoptosis is induced by GAS, and suggest a contributory role of inflammatory caspase-1 to the overall level of GAS-induced macrophage apoptosis; however, distinction from pyroptosis is provided below in studies of inflammatory cytokine production.

Streptolysin O is necessary and sufficient for GAS-induced macrophage apoptosis. Studies of GAS interactions with epithelial cells have implicated various bacterial factors potential agents of apoptosis, including cysteine protease (SpeB), pore-forming cytolysin streptolysin O (SLO), and nicotinamide dehydrogenase (NADase) (Bricker *et al.*, 2002; Kuo *et al.*, 1999; Nakagawa *et al.*, 2001; Tsai *et al.*, 1999). To ascertain the contributory role of specific virulence factor(s), we compared the relative abilities of a panel of isogenic, single-gene deletion mutants to the WT parent M1T1 GAS strain in the macrophage apoptosis assay (**Fig. 4.10**). Elimination of the cell-wall anchored antiphagocytic M1 protein, the surface-expressed hyaluronic acid capsule, potent β-hemolysin/cytolysin streptolysin S, the phage-encoded DNAse Sda1, or the cysteine protease SpeB did not significantly diminish GAS-induced macrophage apoptosis. In contrast, an isogenic mutant lacking cytolysin SLO (ΔSLO) was dramatically reduced in its ability to produce apoptosis (**Fig. 4.10**). Single gene complementation of the isogenic ΔSLO knockout mutant with the *slo* gene on a multicopy plasmid restored the macrophage apoptosis-inducing phenotype even beyond WT levels (**Fig. 4.11**). Heterologous expression of the GAS *slo* gene in the nonpathogenic LL conferred a strong pro-apoptotic phenotype, indicated the

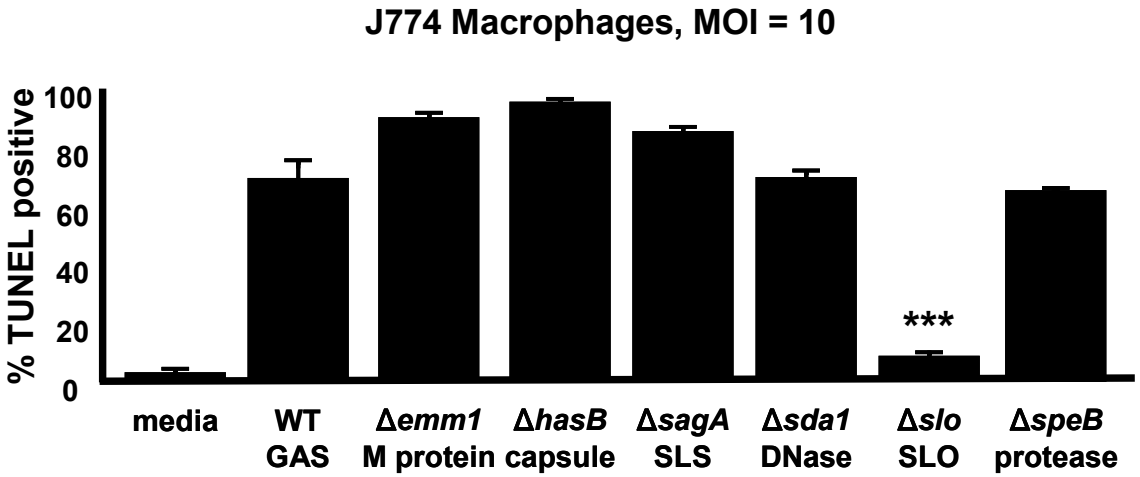

Figure 4.10 Screen of isogenic GAS virulence factor mutants reveals streptolysin O (SLO) is necessary for induction of macrophage apoptosis.

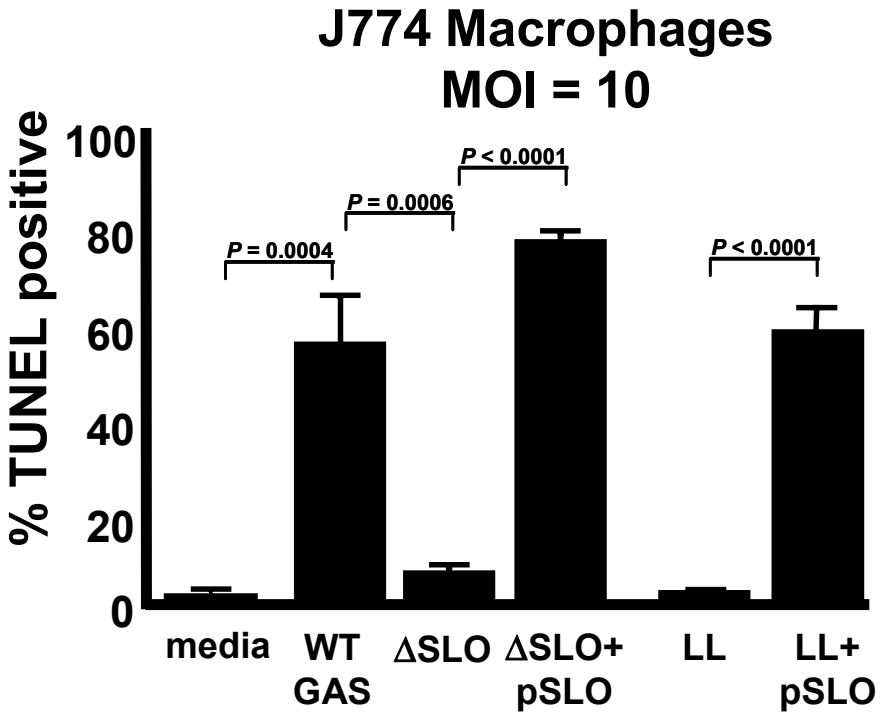

Figure 4.11 Single gene complementation of SLO rescues the mutant and SLO confers to *L. lactis* the ability to induce macrophage apoptosis.

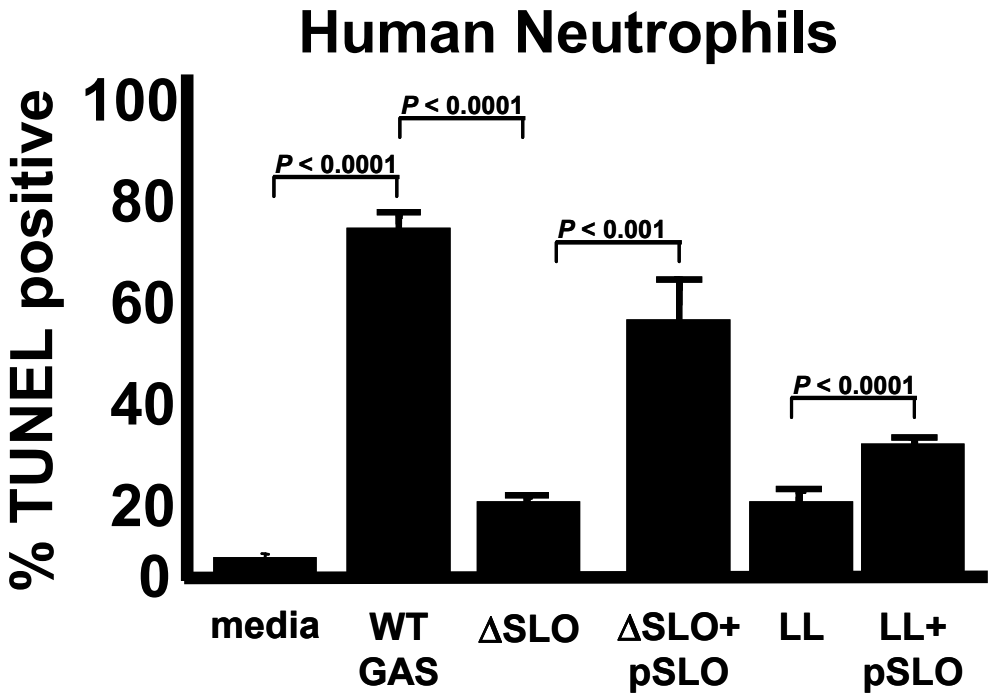

Figure 4.12 SLO is also necessary in GAS and sufficient in *L. lactis* for induction of neutrophil apoptosis.

toxin was not only necessary but also sufficient to trigger macrophage programmed cell death (**Fig. 4.11**). Parallel studies using purified human neutrophils (**Fig. 4.12**) indicate that SLO is the GAS virulence factor responsible for the striking phenotype of accelerated neutrophil apoptosis induced by GAS infection (Kobayashi *et al.*, 2003). A direct link of SLO expression to activation of effector caspases-3/7 was confirmed in fluorescent substrate assays comparing lysates from macrophages infected with WT GAS vs. the ΔSLO mutant (**Fig. 4.13a**) or LL expressing the GAS *slo* gene vs. empty vector control (**Fig. 4.13b**).

SLO has been implicated in the generation of membrane pores that allow specific transport of GAS NADase into host cells (Madden *et al.*, 2001). An isogenic knockout of the *nga* gene encoding GAS NADase (ΔNGA) was reduced in its ability to induce macrophage apoptosis, but not as markedly as the GAS ΔSLO mutant (**Fig. 4.14**). In contrast to SLO, heterologous expression of NADase in LL was insufficient to confer a proapoptotic phenotype in the macrophage infection assay (**Fig. 4.14**).

Addition of purified SLO protein to macrophages was sufficient to induce dose-dependent macrophage apoptosis (**Fig. 4.15**). Penicillin-killed GAS, unable to induce apoptosis on its own (**Fig. 4.3**), significantly enhanced the apoptosis induced by the purified SLO (**Fig. 4.15**), indicating an essential function of the pore-forming cytolysin can then further synergize with bacterial components in pro-apoptotic signaling.

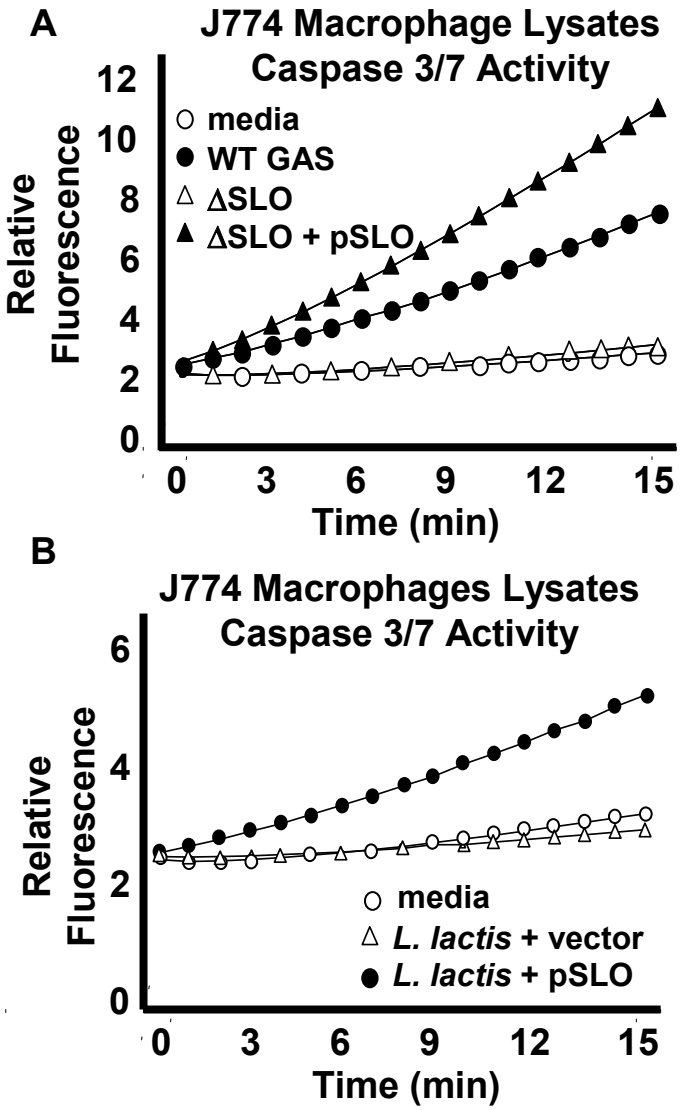

Figure 4.13 (a) Caspase-3 and -7 activation in cell lysates of macrophages infected with WT GAS strains, but not the ΔSLO mutant as assessed by cleavage of fluorescent substrates. (b) SLO expression in *L. lactis* is sufficient to induce caspase-3 and -7 activity in macrophage lysates.

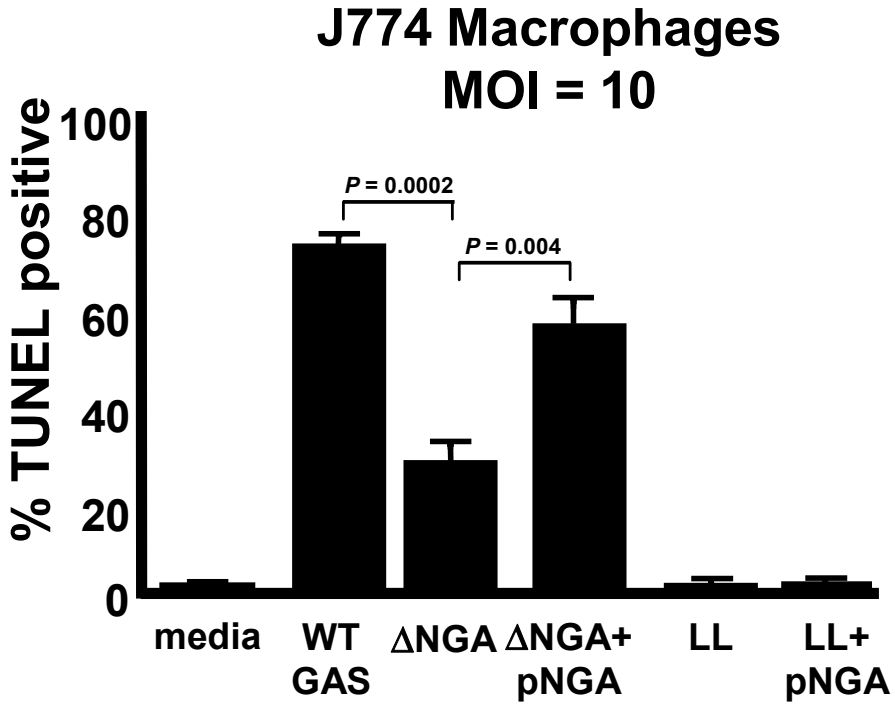

Figure 4.14 The streptococcal NADase (*nga*) plays a contributory, but not sufficient, role in induction of macrophage apoptosis.

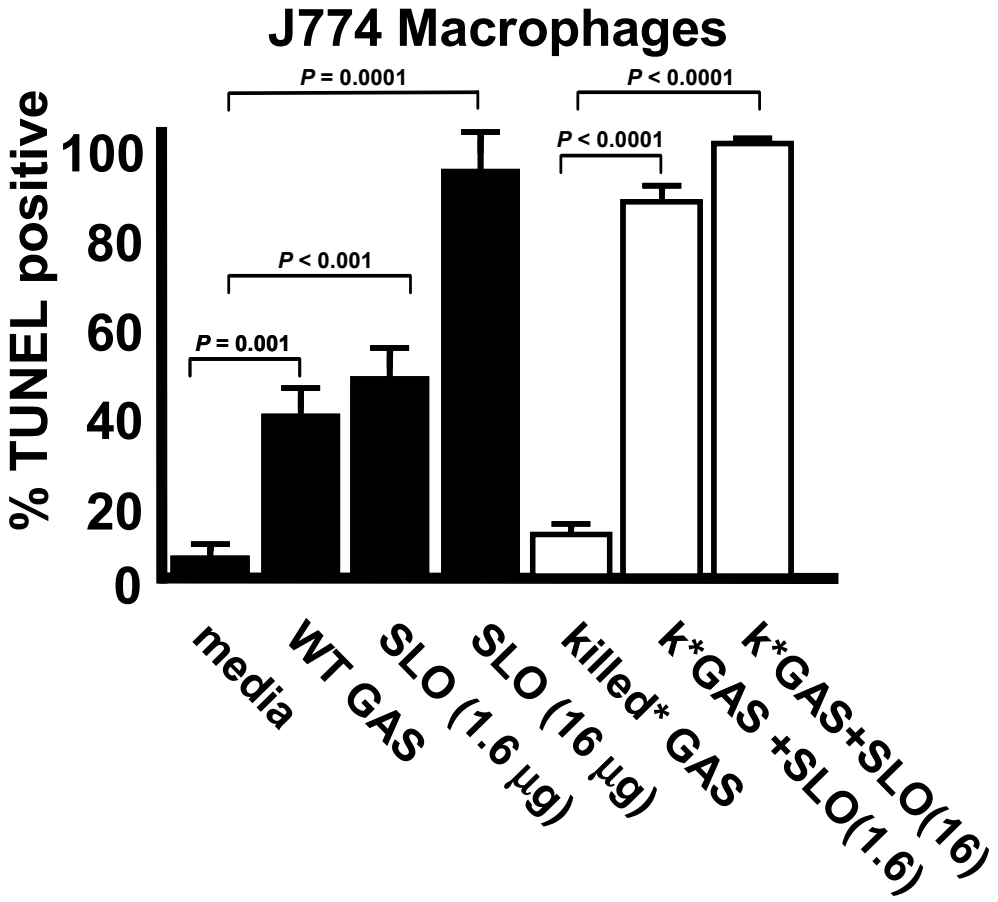

Figure 4.15 Purified recombinant SLO induces dose-dependent macrophage apoptosis, which can be enhanced by the addition of bacterial components.

SLO reduces mitochondrial membrane potential and triggers cytochrome* c *release. A hallmark of apoptosis mediated through the intrinsic pathway of caspase activation is the release of cytochrome *c* from the mitochondria into the cytosol (Liu *et al.*, 1996) where it interacts with Apaf-1 and results in the activation of initiator caspase-9 (Riedl and Salvesen, 2007). Release of cytochrome *c* precedes a decrease in mitochondrial membrane potential ($\Delta\psi_M$) reflecting the opening of mitochondrial membrane transition pores. Using the fluorescent stain tetramethylrhodamine ethyl ester (TMRE) that labels healthy polarized mitochondria, we found that macrophages exposed to WT GAS but not the isogenic ΔSLO mutant showed a rapid loss of TMRE staining indicating reduced $\Delta\psi_M$ (**Fig. 4.16**). The action of SLO to diminish $\Delta\psi_M$ was corroborated by complementation of the ΔSLO mutant with the *slo* gene and heterologous expression of the GAS *slo* gene in LL (**Fig. 4.16**).

Next, cell lysates of mouse primary peritoneal macrophages infected with WT GAS or the isogenic ΔSLO mutant were collected at various time points, separated by centrifugation into mitochondrial and cytosolic fractions, and analyzed for cytochrome *c* by Western blot. Increasing amounts of cytochrome *c* were detected in the cytosol of WT GAS-infected macrophages over time, but not those infected with the ΔSLO mutant (**Fig. 4.17a**). Complementation of the ΔSLO mutant, heterologous expression of *slo* in LL, and addition of purified SLO

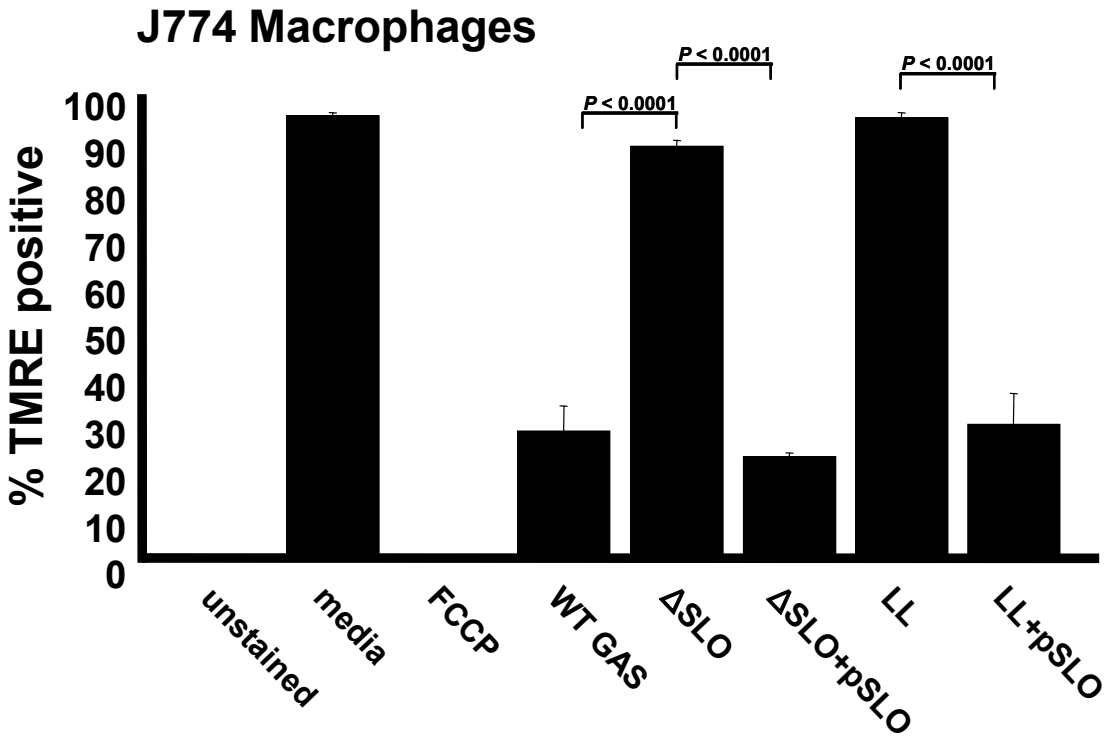

Figure 4.16 SLO-dependent loss of TMRE staining demonstrates perturbation of mitochondrial membrane potential.

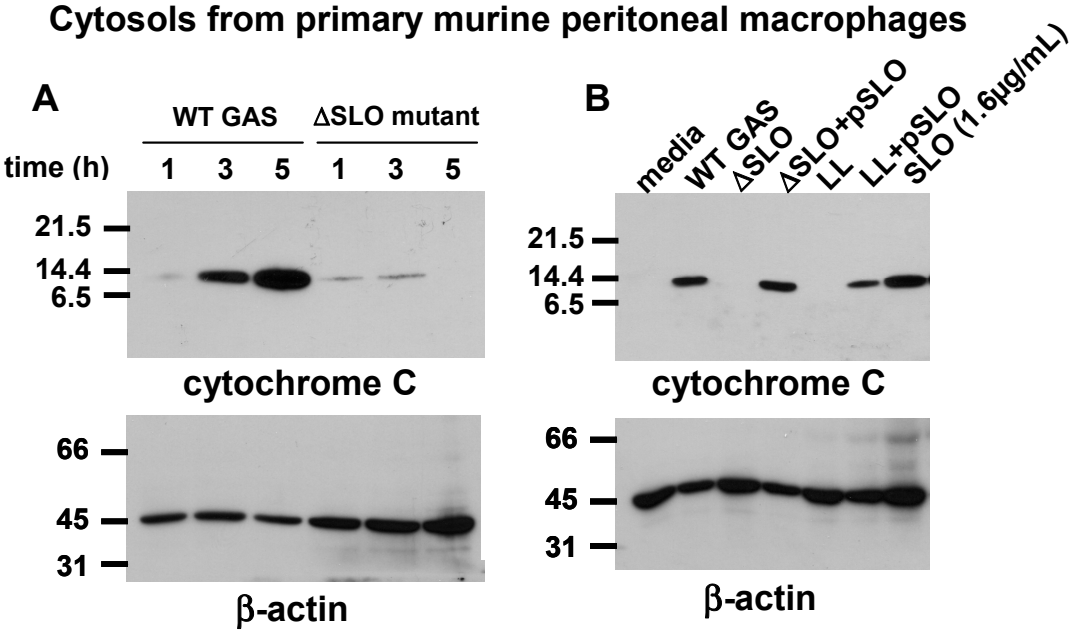

Figure 4.17 Western blot of cytosols of macrophages infected with GAS +/- SLO (a), LL +/- SLO, or purified SLO (b) illustrates SLO-mediated release of cytochrome *c* from mitochondria. Actin serves as loading control.

protein confirmed that the cytolysin was the factor responsible for induction of cytochrome *c* release into the macrophage cytoplasm (**Fig. 4.17b**).

Electron microscopy reveals changes in mitochondrial ultrastructure accompanying apoptosis. A number of studies have revealed changes in mitochondrial structure during apoptosis induced by the intrinsic pathway. These changes include fragmentation of the mitochondria network and remodeling of the inner mitochondrial membrane, the significance of which is debated (Frezza *et al.*, 2006; Scorrano *et al.*, 2002; Sun *et al.*, 2007). Scorrano et al. observed a dramatic inner membrane remodeling in purified rat liver mitochondria treated with the proapoptotic protein, Bid, to induce cytochrome *c* release, and proposed that the remodeling was required for efficient and complete release of cytochrome *c* from the intracristal compartments (Frezza *et al.*, 2006; Scorrano *et al.*, 2002). Subsequently, a correlated three-dimensional light and electron microscopy study of apoptosis induced in HeLa cells by etoposide observed a transformation of the mitochondrial inner membrane in normal mitochondria into one in which the inner membrane fragmented into numerous individual vesicular matrix compartments. The changes in mitochondrial inner membrane structure have been attributed to changes in amount and processing of the dynamin related protein, OPA1 (mgm1p in yeast) that is required for mitochondrial inner membrane fusion (Arnoult *et al.*, 2005; Frezza *et al.*, 2006; Griparic *et al.*, 2004). Sun et al. found that inhibition of caspases efficiently inhibited all changes in the inner membrane conformation without inhibiting release of cytochrome *c*.

In order to investigate apoptosis induced by GAS infection of macrophages, we examined four samples by electron microscopy of thin sections. Mitochondria in macrophages treated with media contained, as expected, only mitochondria with normal morphology (**Fig. 4.18a**), which represents over 95% of 357 mitochondrial images observed in 21 cells. In macrophages infected with GAS, mitochondrial structure was altered; fewer than a quarter of the 300 mitochondrial images observed in 23 cells appeared normal while approximately half displayed the condensed matrix appearance of the mitochondrion (**Fig. 4.18c**) and the remainder displayed dilated cristae (**Fig. 4.18b**). Inhibition of caspases with Q-VD-OPH efficiently inhibited these structural changes, and nearly 90% of over 300 mitochondria observed in 26 cells had normal inner membrane morphology. On the other hand, treatment of macrophages with SLO produced the same morphological changes as infection with GAS with even fewer normal mitochondria and somewhat more with condensed matrix compartments or dilated cristae. Furthermore, the samples infected with GAS and those treated with SLO contained a significant number of cells that have lysed that were not observed in the control treated with media. The GAS infected cells treated with Q-VD-OPH also contained some lysed cells but fewer than those infected with GAS alone or treated with SLO.

SLO blunts macrophage immune response and contributes to GAS virulence. The rapid nature of GAS SLO-induced apoptosis suggested the

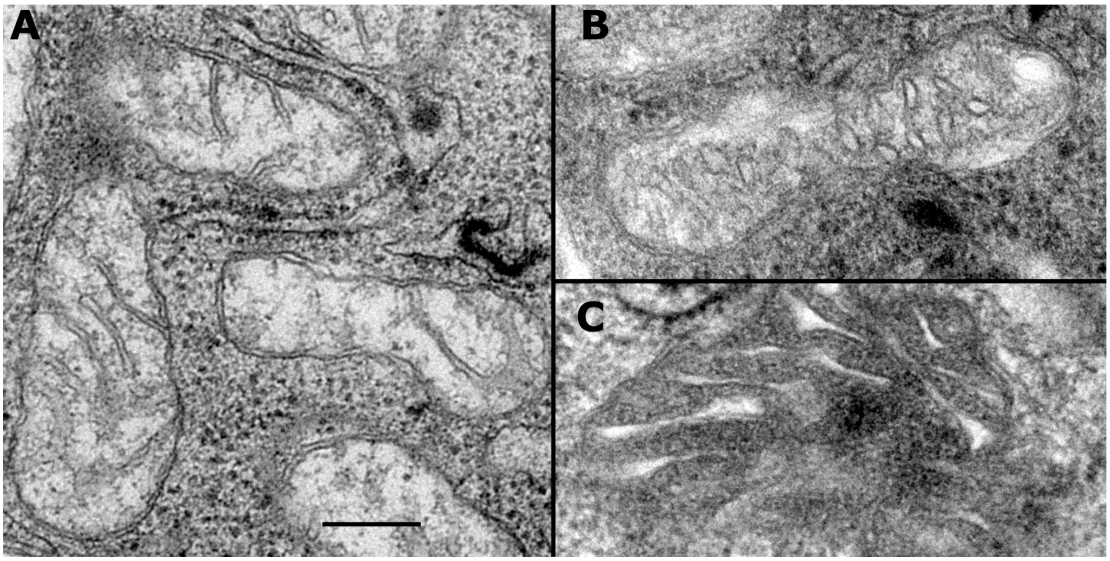

Figure 4.18 Mitochondria in control cells treated with media display typical ultrastructure with the inner membrane projecting into the matrix at crista junctions to form lamellar cristae (a). Mitochondria in GAS-infected cells show altered cristae structure consistent with swollen crista compartments (b). Some of the mitochondria in GAS-treated cells have a different appearance with irregularly-shaped cristae cross-sections within a very dense matrix compartment (c). Scale bar is 200 nm.

process could adversely impact macrophage function in innate immune response activation. When BMD macrophages from C57B/6 mice were infected by the WT M1T1 GAS, the isogenic ΔSLO mutant, and the complemented mutant, we found a pattern of rapid SLO-dependent apoptosis resembling that seen in the J774 cell studies (**Fig. 4.19**). Tumor necrosis factor alpha (TNFα) and interleukin-1β (IL-1β) are key inflammatory cytokines that contribute to broader immune system activation in response to pathogens. When TNFα and IL-1β release from infected macrophages was measured by ELISA, the levels detected were far more than 3-fold greater in macrophages exposed to the ΔSLO mutant than to the WT strain or complemented mutant (**Fig. 4.20**). To exclude whether this difference was a result of increased cytokine gene expression in macrophages infected with the ΔSLO mutant, we performed quantitative real time RT-PCR of macrophage cytokine transcripts. TNFα and IL-1β mRNA increased over 200-fold and 1,000-fold respectively in macrophages infected with WT GAS, and these levels were not increased, but rather slightly decreased, in macrophages infected with the ΔSLO mutant (**Fig. 4.21**). SLO was also seen to blunt the early cytokine response to M1T1 GAS infection *in vivo*; in C57B/6 mice challenged by intravenous injection, serum TNFα levels 6h post-infection were significantly lower in animals challenged with the WT strain than those challenged with the isogenic ΔSLO mutant (**4.22**).

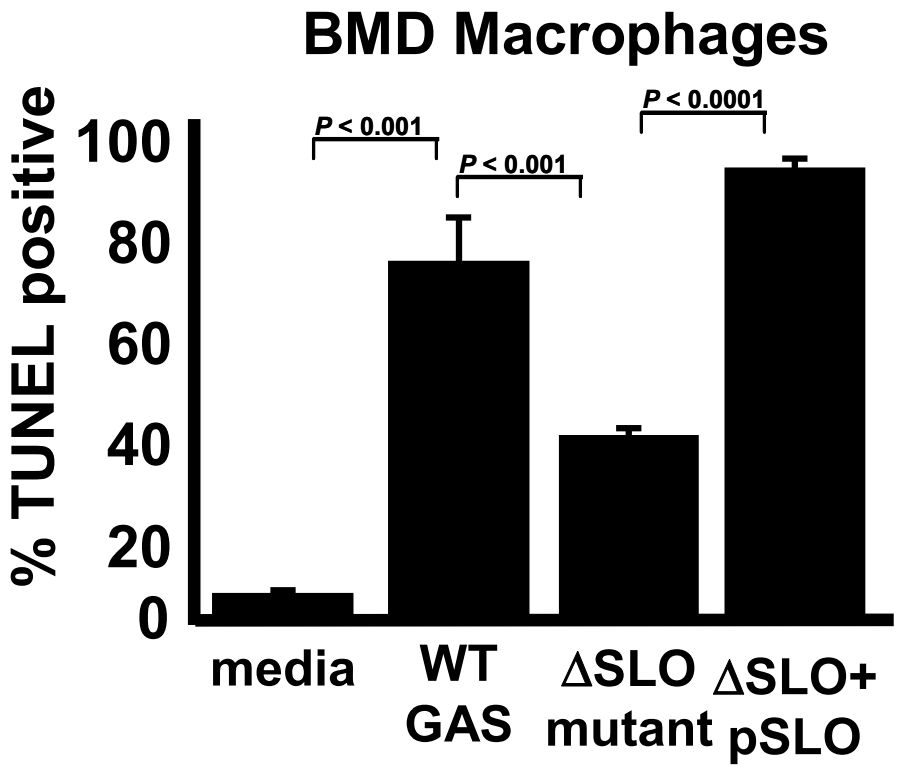

Figure 4.19 Role for SLO in apoptosis induction observed upon GAS infection of primary bone-marrow derived macrophages.

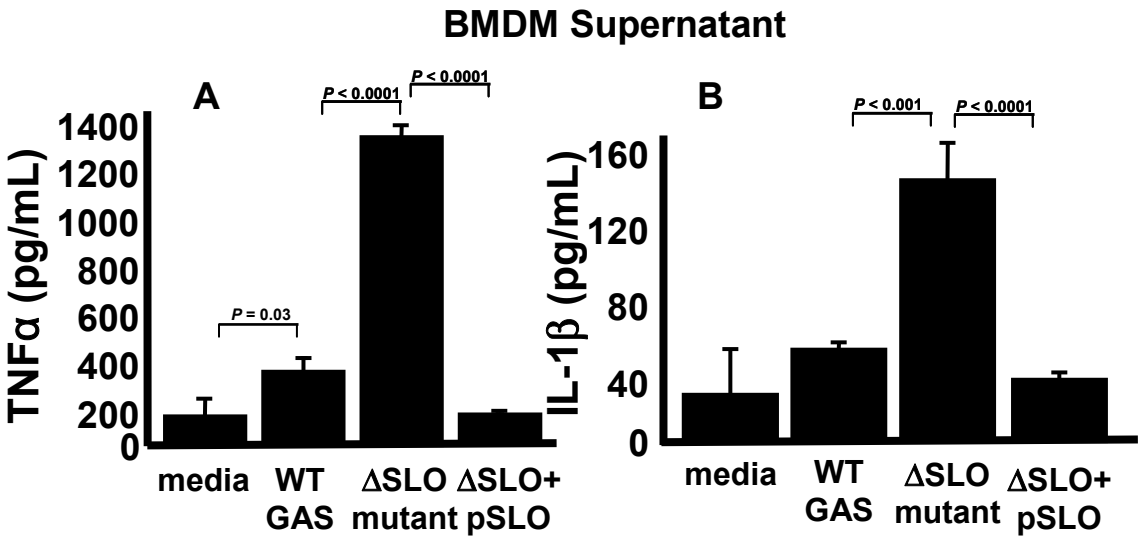

Figure 4.20 Reduced TNFα (a) and IL-1β (b) levels in supernatants of primary mouse macrophages infected with SLO-expressing strains.

Furthermore, we found that macrophage apoptosis mediated by SLO contributed directly to GAS evasion of macrophage killing. Upon coincubation with J774 macrophages, the WT GAS strain survived better than the isogenic ΔSLO mutant (**Fig. 4.23**). Corroborating a role for accelerated apoptosis in this survival phenotype, addition of the pan-caspase inhibitor Q-VD-OPH to retard apoptosis enhanced the ability of the macrophages to kill the WT GAS strain (**Fig. 4.24**). Ultimately, impairment of host macrophage innate immune response correlated with increased virulence of the WT GAS M1T1 strain in the mouse intraperitoneal infection model. Compared to the ΔSLO mutant, the WT parent strain reached significantly higher bacterial loads at 24 h (**Fig. 4.25**) and produced greater overall mortality (90% vs 40% by day 5) (**Fig. 4.26**). Thus SLO, one of the most strongly upregulated genes *in vivo* during M1T1 GAS infection (Sumby *et al.*, 2006), is an important virulence factor in the pathogenesis of systemic infection, acting to impair macrophage bactericidal function and cytokine responses.

DISCUSSION

Our data demonstrate that live GAS induce rapid and significant dose-dependent macrophage apoptosis upon phagocytosis by macrophages. This cell death is induced by the pore-forming protein toxin SLO, which is both necessary and sufficient for this pathogenic phenotype, and is expressed by all GAS strains. We further identify SLO as the specific virulence determinant responsible for marked neutrophil apoptosis observed upon co-incubation with GAS compared to

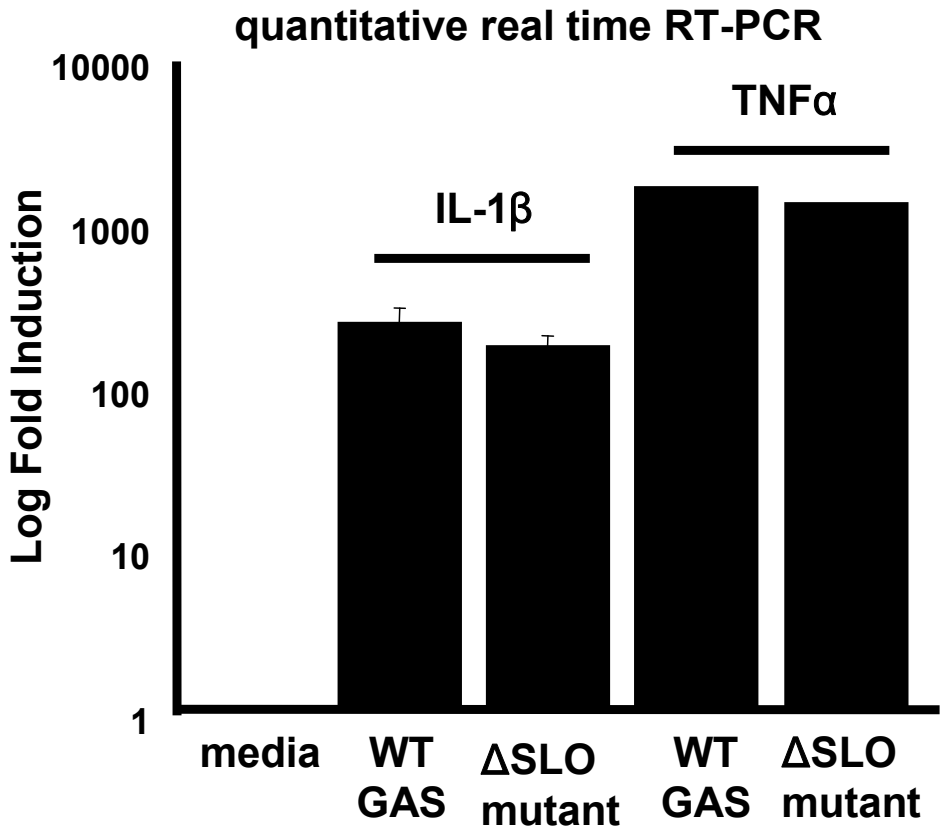

Figure 4.21 TNFα and IL-1β gene transcripts are significantly upregulated by infection with both WT GAS and the ΔSLO mutant.

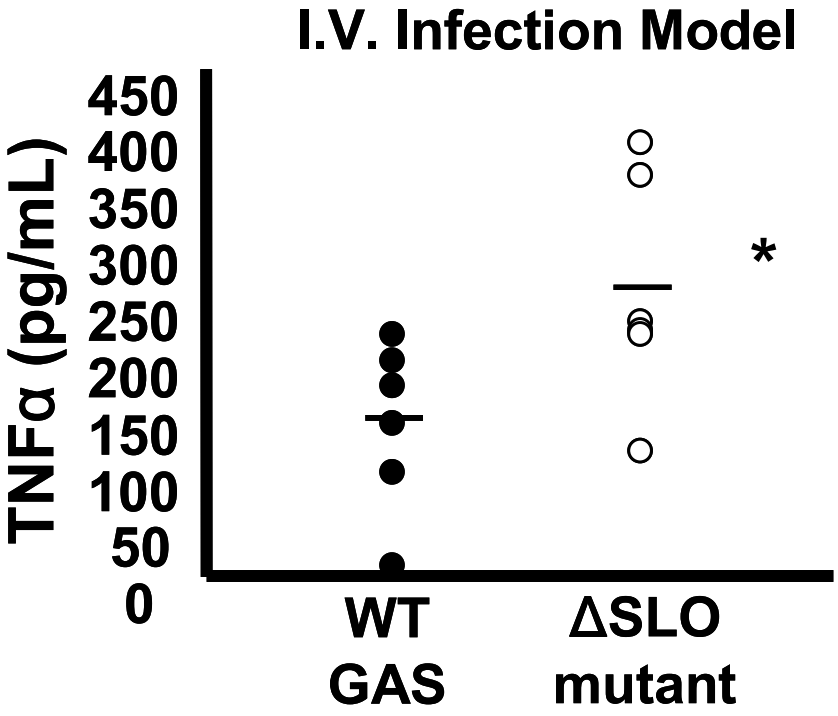

Figure 4.22 Decreased TNFα detected in the blood of mice 6 h after intravenous infection with WT GAS vs. the ΔSLO mutant.

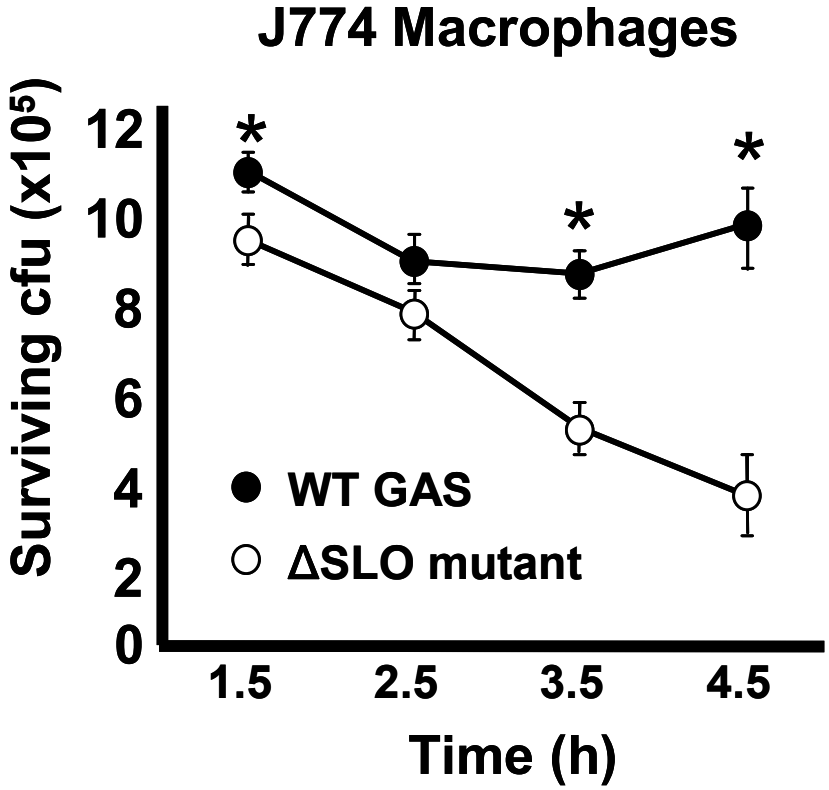

Figure 4.23 Higher survival of WT GAS vs. ΔSLO mutant in the presence of macrophages.

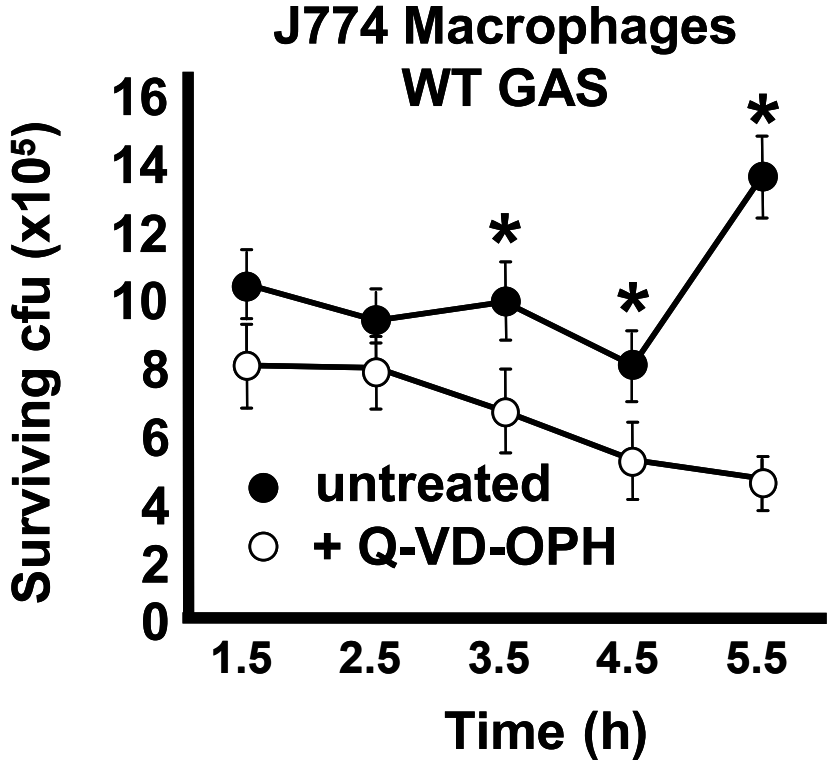

Figure 4.24 Caspase inhibitor Q-VD-OPH enhances the ability of macrophages to kill WT GAS.

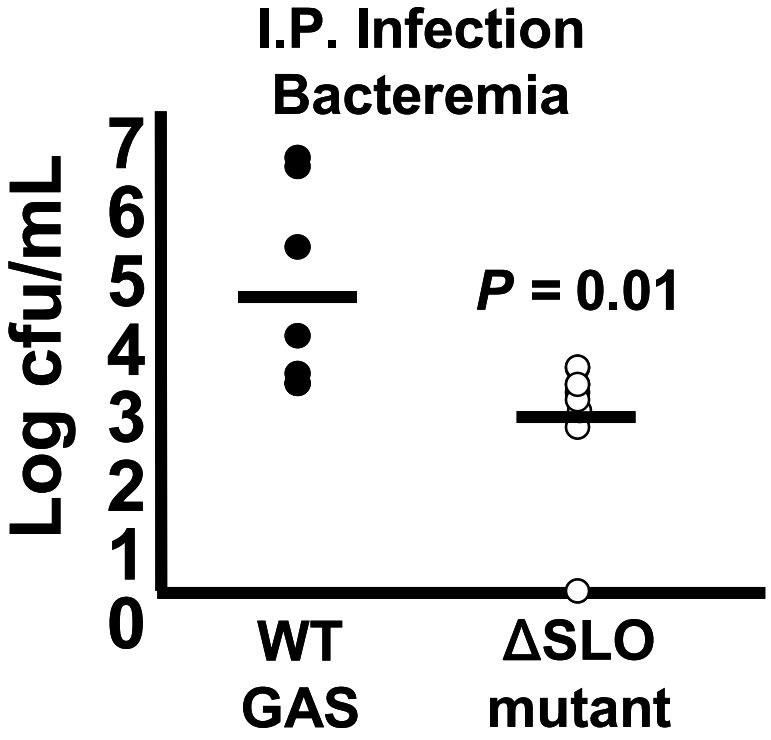

Figure 4.25 Higher numbers of WT GAS than ΔSLO mutant recovered from blood of mice injected intraperitoneally. t = 24 h.

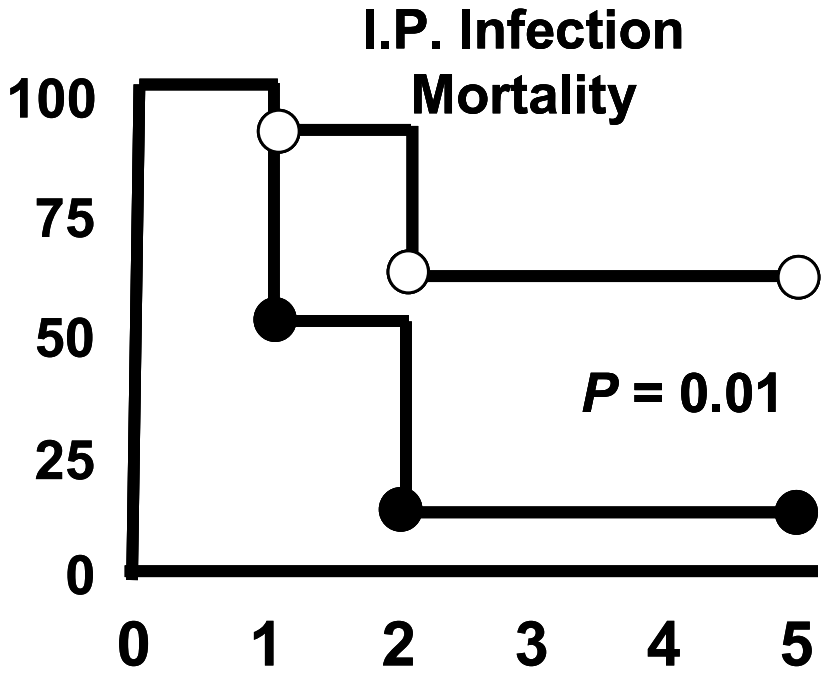

Figure 4.26 The ΔSLO mutant is significantly attenuated (60% survival) vs. the WT GAS (10% survival) in a mouse systemic infection model.

other bacteria (Kobayashi *et al.*, 2003), identifying a mechanism by which the pathogen may overcome both major classes of human phagocytic cells. The rapid proapoptotic potential of SLO appears unique and specific, since induction of macrophage apoptosis by GAS was not reduced upon elimination of the potent membrane active toxin SLS. GAS NADase contributes to the magnitude of macrophage apoptosis, but itself is not sufficient to produce the phenotype, likely due to the observation that NADase is dependent on SLO-induced pores for membrane translocation (Madden *et al.*, 2001). By depleting intracellular energy stores, the NADase may diminish the host's ability to combat or repair the membrane toxic effects of SLO (Michos *et al.*, 2006).

Some previous studies of GAS interaction with epithelial cells assessed apoptosis as an outcome variable. In respiratory epithelial cell lines, internalization of the bacteria was observed to be critical for induction of apoptosis phenotype (Nakagawa *et al.*, 2001) and a provocative role was identified for the GAS cysteine protease, SpeB (Tsai *et al.*, 1999). In contrast, internalization was said to inhibit GAS-induced apoptosis of keratinocytes, where the phenotype was attributed to SLO-dependent influx of extracellular calcium (Cywes Bentley *et al.*, 2005). In our GAS macrophage infection models, we find no requirement for cysteine protease SpeB, and show that cytochalasin D inhibition of bacterial uptake markedly reduces apoptosis. These findings are consistent with the observed involvement of the inflammasome and caspase-1,

which rely on intracellular signaling for activation, and suggest an intracellular target for SLO membrane toxicity may participate in apoptosis induction.

We established that GAS-induced macrophage apoptosis is triggered by the activation of caspases, including a significant contributory role for inflammatory caspase-1. Gurcel et al. demonstrated that another bacterial pore-forming toxin, aerolysin, stimulated the activation of caspase-1, with the net effect of promoting cell survival in the presence of the purified toxin (Gurcel *et al.*, 2006). Conversely, we find that in the context of live infection with GAS expressing SLO, inhibition of caspase-1 results in reduced apoptosis and increased macrophage survival. The opposite results may reflect the small size (1-2nm) of aerolysin-induced pores (Mota *et al.*, 2005), compared to the much larger size (25-30 nm) of pores produced by SLO and other cholesterol-dependent pore-forming toxins (Shatursky *et al.*, 1999), which could more rapidly effect intracompartmental fluxes of a larger array of host cell and bacterial components.

GAS-induced macrophage apoptosis differs significantly from another form of programmed cell death induced by *Salmonella* and *Shigella*, termed pyroptosis (Boise and Collins, 2001; Cookson and Brennan, 2001). Pyroptosis is an inflammatory cell death completely dependent on caspase-1 and resulting in a strong inflammatory cytokine response (Fink and Cookson, 2005). Our ELISA data, *in vitro* and *in vivo*, contradict the possibility that GAS also induces

pyroptosis, since we observed very low levels of inflammatory cytokines released from the macrophages dying following infection with WT GAS. Our quantitative PCR data demonstrate that macrophages are activated to markedly increase expression of the corresponding cytokines in response to WT (and ΔSLO) GAS infection, so low levels found in the WT infected cells are due to post-transcriptional events, namely accelerated apoptosis. The blunting of the macrophage-elicited systemic immune response to the invading bacterium reveals a novel virulence mechanism for GAS to undermine important host leukocyte functions.

Based on the knowledge that GAS must be internalized to induce apoptosis, and the SLO-dependent release of cytochrome *c* from the mitochondria, we propose that SLO effects accelerated macrophage apoptosis by pore formation in the membranes of the endocytic vesicle followed by direct attack of SLO upon the mitochondrial outer membrane, leading to release of proteins, including cytochrome *c,* into the cytosol, activating the intrinsic pathway of apoptosis. This is supported by electron microscopy macrophages infected with GAS or treated with SLO showing changes in mitochondrial structure characteristic of apoptosis. The observation that apoptosis induced by purified SLO protein is further enhanced in the presence of bacterial components suggests the cooperation of additional pathways of apoptosis induction. SLO pore formation may allow the passage of inflammasome-activating cellular

components into the cell cytosol, synergistically promoting apoptosis through additional caspase-dependent pathways.

Through induction of apoptosis, WT GAS were better able to survive in the presence of macrophages than the isogenic SLO-deficient mutant. This difference found was corroborated *in vivo* where the WT GAS blunted host cytokine responses and demonstrated enhanced bloodstream survival and lethality compared to the toxin-negative strain. Addition of a pan-caspase inhibitor protected macrophages from apoptosis and enhanced GAS killing. A deeper understanding of the pathway of GAS-induced macrophage apoptosis could aid in the identification of novel targets for immunomodulatory therapy of invasive GAS infections, estimated to strike > 600,000 individuals each year worldwide (Carapetis *et al.*, 2005). Neutralization of the SLO toxin production may also represent a virulence factor-based therapy to support innate immune clearance of the pathogen.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Cell Culture Conditions. Serotype M1T1 GAS strain 5448 is an isolate from a patient with necrotizing fasciitis and toxic shock syndrome (Kansal *et al.*, 2000). *Lactococcus lactis* strain NZ9000 is derived from MG1363 and lacks the *nis* operon (Kiupers *et al.*, 1998). WT GAS, isogenic gene deletion mutants, and LL were grown in Todd-Hewitt broth (THB) (Difco); when required, retention of an *slo* or *nga* expression plasmid or empty vector control

was ensured by addition of 2 μg/mL (GAS) or 5 μg/mL (LL) erythromycin to the media. For all *in vitro* and *in vivo* assays, overnight cultures were diluted 1:10, grown to early-logarithmic phase (~2 hours), pelleted, and resuspended to OD_{600nm} = 0.4 (~2 x 10^8 cfu/ml GAS) and diluted to desired concentrations. The murine macrophage cell line, J774, was grown in RPMI supplemented with 10% FBS (Gibco). Primary bone marrow-derived macrophages were prepared as described (Hsu *et al.*, 2004) with slight modification. Bone marrow cells were collected from mice and cultured in DMEM (high glucose) supplemented with 20% L-929 cell conditioned medium for 7 days. The adherent cells (BMDMs) were then collected and cultured in DMEM (high glucose) with10 ng/mL MCSF (Peprotech) overnight before bacterial infection. Human neutrophils were isolated from fresh whole blood (Polymorph Prep) and cultured in RPMI containing 2% autologous serum.

Generation of GAS SLO and NADase Allelic Exchange Mutants. PCR was used to amplify upstream and downstream DNA fragments from the M1 GAS 5448 chromosome immediately flanking the genes of interest. Primers pairs used were: (a) *slo*-upF (5'-gtatcactagaagagactct-3') + *slo*-upR (5'-gtccttcatacctttttatc-3' with 30 bp 5' extension matching 5' end of *cat* gene); (b) *slo*-downF (5'-gactggttcaagagataagc-3' with 30 bp 5' extension matching 3' end of *cat* gene) + *slo*-downR (5'-gacagttggggtcaaatcag-3'); (c) *nga*-upF (5'-gggctggttcgtcctccaaac-3') + *nga*-upR (5'-taaaccaccttatattatt-3' with 30 bp 5' extension matching 5' end of *cat* gene) (d) *nga*-downF (5'-

caatatgtataaggtgccaaaggg-3' with 30 bp 5' extension matching 3' end of *cat* gene) + *nga*-downR (5'-cagtgtgatcttcttcgctc-3'). PCR was performed using the respective upstream and downstream fragments and an amplicon of the *cat* gene to yield a fusion product in which *cat* replaced gene of interest precisely in GAS chromosomal context. This fusion product was T-A cloned into pCR2.1-Topo (Invitrogen), then subcloned into pHY304, a temperature sensitive vector with an erythromycin (Em) resistance marker. These knockout plasmids were transformed into GAS M1 WT 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. Precise, in-frame allelic exchange of *slo* and *nga* with *cat* in the GAS chromosome was confirmed by PCR analysis.

Complementation and Heterologous Expression Studies. The M1 5448 *slo* gene was amplified using primers *slo*F (5'- gcttgatatggtcgaaagaac-3') and *slo*R (5'-ggagtgggcacaaggcctca-3'), and the *nga+ifs* genes using primers *nga*F (5'- gtttctcatgtaaaccacc-3') and *ifs*R (5'-gaacaataaaaacattttag-3'). Each amplicon was T-A cloned into pCR2.1-TOPO (Invitrogen), then subcloned into streptococcal expression vector pDCerm (Jeng *et al.*, 2003), before being introduced by electroporation into GAS mutants or *L. lactis* and transformants identified by Em selection.

Apoptosis Measurement by TUNEL Assay. J774 macrophages were plated at 1.5 x 10^6 cells/well in 12-well plates and BMD macrophages plated at 2 x 10^6 cells per well in 6 well plates one day prior to infection. Neutrophils were plated at 4 x 10^6 cells per well in 6 well plates. Bacteria were grown to log phase, diluted in cell culture media to allow 2 x 10^7 cfu (4 x 10^7 for neutrophil assays) to be added to each well. Plates were centrifuged at 2,000 rpm x 5 min to ensure bacterial contact with cells, then incubated at 37°C, 5% CO_2. One hour after infection, penicillin (5 μg/ml) and gentamicin (100 μg/ml) were added to the media to kill residual extracellular bacteria. At 4 h after infection, cells were collected, fixed, and permeabilized according to the Apo-BrdU TUNEL assay protocol (BD Biosciences). Cells were kept in 70% ethanol at -20°C until they were stained for DNA fragmentation following the TUNEL protocol (BD Biosciences). Apoptosis was quantified by flow cytometry (BD FACSCalibur) with analysis using FlowJo software.

Reagents. All inhibitors were added to cells for 1 h prior to infection and left for the duration of the assay. Equal volumes of DMSO alone had no effect on macrophage apoptosis. Concentrations used were as follows: 5 μg/mL cytochalasin D (Sigma), 200 μM Q-VD-OPH (MP Biomedicals), 20 μg/mL YVAD-CHO (Calbiochem), 1 μg/mL actinomycin D (Sigma), 100 nM tetramethylrhodamine ethyl ester (TMRE) (Invitrogen/Molecular Probes), and 5

μM carbonylcyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) (Invitrogen/Molecular Probes).

Mitochondrial Membrane Depolarization. J774 cells were plated as for the TUNEL assay. Thirty minutes prior to infection with GAS, cells were preloaded with TMRE. Cells were washed once with PBS and then infected as usual. Antibiotics were added at 1 h and at 4 h cells were collected and washed with PBS before assessing fluorescence by flow cytometry (BD FACSCalibur). The mitochondrial membrane uncoupler, FCCP, was added for 2 h as a positive control.

Caspase Activity Assay J774 cells were plated and infected as for the TUNEL assay. At 4 h, cells were collected, washed once with PBS, and resuspended in 100 μl of modified RIPA buffer. After 10 m incubation on ice, samples were centrifuged at full speed and lysates collected and kept on ice. Lysates were diluted in water and mixed with fluorogenic caspase-3/7 substrate DEVD-AFC (synthesized in house) at a final concentration of 100 μM in caspase buffer (Denault and Salvesen, 2008). AFC kinetics were measured immediately using the fmax fluroescent plate reader (Molecular Devices) at 37°C with 405 nm excitation and 510 nm emission. Recombinant caspase-3 was used as a positive control.

Cytokine ELISA. Supernatants were collected at 4 h from the apoptosis assays using bone marrow-derived macrophages. ELISA was done on cell supernatants for TNFα and IL-1β.(R&D Systems). TNFα levels in mouse serum were quantified by ELISA (BD Biosciences, San Diego, CA). Standards were also diluted in serum as a control for mouse experiments.

Quantitative RT-PCR. Primary mouse macrophages at 4 x 10^6 cells/well incubated in RPMI + 0.5% FBS overnight were infected with WT GAS or the ΔSLO mutant at MOI = 10, antibiotics added at 1 h to kill extracellular bacteria. Cells were collected by scraping at 4 h cells and pelleted. RNA was collected using the Qiagen RNeasy Mini kit, cDNA was made using SuperScript III First-Strand Synthesis SuperMix (Invitrogen), followed by quantitative RT-PCR (SYBR GreenER qPCR SuperMix, Invitrogen) using established TNFα, IL-1β and GADPH (control) primer pairs.

Expression and Purification of Recombinant Streptolysin O. The SLO gene was cloned into vector pET15b and transformed into BL21 DE3 *E. coli*. Bacteria expressing SLO were cultured in 3 L of Difco 2xYT medium (BD) and incubated at 37°C with shaking. Expression was induced in cultures at 0.8 OD_{600} with 0.5 mM IPTG (BioVectra), and maintained at 30°C for 4 hours. Bacterial pellets were disrupted by sonication, and soluble 6x histidine-tagged streptolysin-O was purified using Ni-NTA agarose (Invitrogen). Further purification was achieved using Q-sepharose ion exchange chromatography (Pharmacia

Biotech). Proteins were eluted with a salt gradient, and monitored by SDS-PAGE. Fractions corresponding to the full length SLO were pooled, quantitated by A_{280}, and frozen in aliquots at -80°C. Assays were performed in the presence of 10 mM DTT (reducing conditions).

Murine Infection Model. Eight week female C57B/6 mice were injected intraperitoneally (i.p.) with 3.3 x 10^7 cfu suspended in 100 μl PBS of GAS WT or the isogenic ΔSLO mutant. Blood was collected at 24 h and plated to quantify bacterial load. Survival was monitored for 5 days. For intravenous infections, C57B/6 mice were injected i.v. with 1 x 10^8 cfu and blood was collected at 6 h. Blood was plated for quantification of bacterial load, and centrifuged at 500 x g to separate serum which was frozen for ELISA.

Macrophage Killing Assays. J774 cells were seeded at 7.5 x 10^5 cells per well in 24 well plates the day prior to the assay. Bacteria were added at an MOI of 10 in 350 μl of assay media (RPMI 2% FBS) and plates centrifuged at 2000 rpm to ensure contact of the bacteria to the macrophages. Plates were incubated for 30 m at 37°C, 5% CO_2, and then penicillin (5 μg/ml) and gentamicin (100 μg/ml) were added to each well to kill extracellular bacteria and the plates incubated for an additional 1 h. Each well was then rinsed 1x with PBS to remove extracellular dead bacteria, and 350 μl of fresh assay media was added to each well. This was the 1.5 h time point. At various time points, cells were pipetted off

wells and transferred with supernatants to 1.5 ml eppendorf tubes. Cells and bacteria were pelleted at 7,000 rpm for 5 minutes and supernatants removed. Pellets were lysed using 0.025% triton-X and dilutions plated to quantify bacteria.

***Electron* Microscopy.** J774 macrophages were plated at 10^7 cells in 10 cm tissue culture plates. The following day, plates were treated with media control, GAS (MOI 20), 200 μM Q-VD-OPH and GAS (MOI 20), or 16 μg/ml of purified recombinant SLO. Antibiotics were added at 1 h and cells collected and pelleted at 4h. Pellets were then fixed on ice in a 2.5% paraformaldehyde-2.5% glutaraldehyde-0.1 M sodium cacodylate pH 7.4 buffer at least overnight. After washing 3 times in ice-cold 0.1 M sodium cacodylate buffer containing 2.5% sucrose, fixed cells were incubated with 1% osmium tetroxide in acetate-veronal solution (Palade, 1952) for one hour on ice in the dark. After washing with acetate-veronal solution 3 times for 5 minutes each, fixed cells were stained and stabilized en bloc in 0.5% uranyl acetate in acetate-veronal solution pH 6 overnight at room temperature in the dark. Cells were then rinsed with ddH_2O and dehydrated in an ice-cold ethanol series of 70, 90, 100% successively on ice for 5 minutes each. The cells were then washed 3 times for 5 minutes each in fresh 100% ethanol and 3 times for 5 minutes each in fresh 100% acetone at room temperature. The cells were next infiltrated in 67% ethanol-33% epon for 3 hours with agitation at room temperature followed by 34% ethanol-66% epon overnight with agitation at room temperature. The next day, the cells were infiltrated in 100% epoxy epon with agitation at room temperature for 24 hours

after which the samples were placed in an oven and allowed to polymerize in 100% epon blocks at 60°C for 48 hours. Thin sections (approximately 70 nm) were stained with uranyl acetate and lead citrate before examination in an FEI Tecnai 12 TEM. Images were recorded on a Teitz 214 digital camera.

Statistical analyses All statistics were performed using the Student's t-test; *P* values < 0.05 were considered significant. Kaplan-Meyer statistics were applied to the mouse survival curve.

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Chapter V

Conclusions and Perspectives

INTRODUCTION

Group A *Streptococcus* (GAS) is a leading human pathogen responsible for a wide variety of diseases. There are hundreds of millions of cases annually of non-invasive GAS infections such as pharyngitis and impetigo (Carapetis *et al.*, 2005) leading to a huge economic burden in the United States alone (Pfoh *et al.*, 2008). More invasive GAS diseases include toxic shock syndrome, necrotizing fasciitis, and rheumatic heart disease. The global incidence of these diseases is almost 20 million (Carapetis *et al.*, 2005), indicating a need for strategies for treatment and prevention. An understanding of the molecular mechanisms of disease pathogenesis is necessary to elucidate novel therapeutic approaches to the management of GAS infections. Hopefully the work contained here has broadened our understanding of GAS virulence mechanisms which will aid in the development of therapies.

SOF-MEDIATED INVASION OF EPITHELIAL CELLS

Serum opacity factor (SOF) is a multidomain protein produced by approximately half of GAS strains. Its characteristic fibronectin binding domain has been previously reported to play a role in binding host fibronectin and fibrinogen (Courtney *et al.*, 1999; Rakonjac *et al.*, 1995). SOF has recently been shown to contribute to resistance to phagocytosis (Courtney *et al.*, 2006), a novel function for this protein. Here we report that SOF plays a critical role in epithelial cell invasion, a niche which may protect the bacteria from immune cell recognition and antibiotic therapy, in addition to being the first step in causing

invasive disease. Surprisingly, we elucidated that the activity domain of SOF, responsible for the opacification of mammalian serum, plays a significant role in invasion independent of the fibronectin binding domain.

SOF is co-transcribed on an operon with streptococcal fibronectin binding protein X (SfbX), a gene which shares homology to SOF's fibronectin binding domain, but has an N-terminal domain of unknown function. Our data demonstrates that SfbX is dispensable for epithelial cell invasion and virulence in a murine model of. We postulate that SfbX must be serving some function for the bacteria to continue to express it, but no reports have been published. Jeng et al. (Jeng *et al.*, 2003) noted a stretch of SfbX bearing some homology to the *Staphylococcus aureus* coagulase, dysregulation of coagulation being a well-known symptom of sepsis. It would be interesting to investigate if SfbX is playing a role in coagulation.

Our data utilizing isogenic mutants and heterologous expression has demonstrated a clear role for the activity domain of SOF in epithelial cell internalization. Based on the dose-dependent inhibition of GAS internalization by the addition of an SOF activity domain peptide, we hypothesize that there is a specific host cell receptor that SOF is interacting with to facilitate bacterial uptake. The addition of exogenous peptide may have competitively bound this receptor, interfering with the bacteria's ability to bind and invade. This also argues against the enzymatic activity of SOF mediating invasion. The fact that

not all GAS strains express SOF, including some of the most invasive phenotypes such as M1, may be explained by a specificity for a host cell receptor only found on specific cells types, leading to differential sites of infection for SOF(+) and (-) negative strains. Our heterologous expression of SOF in the M1 serotype led to a significant increase in the ability of this virulent strain to invade epithelial cells, suggesting that this is not a niche M1 excels in. The enormous increase in invasion by *L. lactis* expressing SOF is most likely due to the fact that WT *L. lactis* has no fibronectin binding potential or invasiveness, so the expression of this one protein makes a more significant difference.

The SOF operon has been postulated to have been in all strains at one time, having been eliminated from approximately half of GAS strains. This could be due to the fact that SOF is highly immunogenic (Courtney *et al.*, 2003). The bacteria must maintain a balance between the expression of virulence factors and the attention their antigenicity draws to the bacteria. Elimination of SOF in highly invasive strains may be a requirement for circumventing host defenses during invasive disease. Interestingly, Courtney et al. recently reported a novel function for SOF as an antiphagocytic factor (Courtney *et al.*, 2006). This demonstrates cell-specific interactions for SOF, inducing internalization by epithelial cells, yet resisting it by macrophages. Perhaps the epithelial cell surface molecule involved in invasion is not present on macrophages, as internalization into macrophages may lead to intracellular killing.

Our screen of 45 clinical isolates, both SOF(+) and (-), revealed a correlation between the expression of SOF and invasiveness across many serotypes. It would be interesting to gather information on the types of infections that each isolate caused and look for patterns related to SOF expression. Previous reports have stated that SOF(+) strains have a propensity for skin infections (Bisno and Stevens, 1996) which could be explained by our data. This is supported by the fact that SOF seemed to play a small role in a murine systemic infection with M49 GAS, but a much more significant role in a skin infection model of NF.

In summary this work elucidated a unique function for the opacification domain of SOF in the invasion of epithelial cells and revealed a role for SOF in virulence in a mouse model of NF.

SPYCEP INHIBITION OF NEUTROPHIL MIGRATION AND ACTIVATION

GAS elaborates a variety of proteins that facilitate its invasiveness and immune evasion (Kwinn and Nizet, 2007). Defense against neutrophil killing is mediated through resistance to phagocytosis by classic GAS virulence factors including the M protein and hyaluronic acid capsule. Pore-forming toxins streptolysin S and streptolysin O also offer protection against these immune cells, as well as many genes involved in resistance to killing by cationic antimicrobial peptides (AMPs). Neutrophils release matrices of DNA, histones, and AMPs to trap and kill bacterial extracellularly (Brinkmann *et al.*, 2004).

Recent work has shown that GAS secretes a DNase to degrade neutrophil extracellular traps (NETs) and resist killing by neutrophils (Buchanan *et al.*, 2006; Sumby *et al.*, 2005).

Neutrophils migrate throughout the host until detecting signals released by endothelial cells and macrophages that attract them to site of infections. These signals, or chemokines, form gradients that induce neutrophil extravasation and recruitment. One of the most potent neutrophil chemoattractants is interleukin-8 (IL-8). In this work, we investigate the degradation of IL-8 to avoid eradication by neutrophils. A striking scarcity of neutrophils had been noticed at the site of several necrotizing fasciitis infections, consistent with an insufficiency of chemokines (Cockerill *et al.*, 1998; Hidalgo-Grass *et al.*, 2004). We establish molecular Koch's postulates for the IL-8 protease SpyCEP in delaying neutrophil recruitment and activation.

The inhibition of neutrophil recruitment to sites of infections is the first step in immune evasion. The additional virulence mechanism to avoid killing by neutrophils are secondary; eliminating neutrophil interactions with GAS altogether plays a key role in virulence in a murine necrotizing fasciitis model of infection. Our data demonstrates that SpyCEP effectively cleaves IL-8 to reduce neutrophil migration, and is sufficient for this phenotype when expressed in a non-pathogenic bacterium. The additional functions of IL-8 in promoting NET

release and delaying neutrophil apoptosis are also hampered by expression of the SpyCEP protein.

The reported 25-fold upregulation of SpyCEP expression in invasive isolates (Sumby *et al.*, 2006) not only demonstrates the importance of this virulence factor in GAS pathogenesis, but also highlights that high expression of SpyCEP at early stages of infection may be detrimental to the bacteria. Our data measuring adherence to and invasion of epithelial cells may provide an explanation for the transcriptional regulation of SpyCEP expression. GAS lacking SpyCEP exhibited significantly increased adherence and invasion of epithelial cells, demonstrating that SpyCEP expression early in infection could interfere with initial attachment and colonization.

The bacteria's need to inhibit the recruitment of leukocytes to the sites of infection, and the significant role this played in an *in vivo* model of necrotizing fasciitis, signifies the importance of neutrophils in fighting GAS infections. Neutralization of the SpyCEP protease could be an alternative therapeutic strategy, as this would allow our immune cells to fight the infection instead of using an antibiotic therapy to directly kill the bacterium.

STREPTOLYSIN O INDUCTION OF MACROPHAGE APOPTOSIS

In addition to the hampering of neutrophil recruitment, GAS must elaborate proteins involved in dealing with leukocytes that are present at the sites

of infection and evading killing by these immune cells. The expression of the surface M protein and hyaluronic acid capsule offer the bacteria some protection against phagocytosis, but our data demonstrates that GAS have further defense mechanism in the event that they are internalized.

We elucidated that GAS induces significant and accelerated macrophage apoptosis upon phagocytosis. This cell death is mediated through caspases, including a role for inflammasome activation and caspase-1. The potent toxin streptolysin O (SLO) is necessary for induction of macrophage apoptosis, and purified SLO is sufficient to induce this phenotype. SLO triggers a disruption of mitochondrial membrane potential, ultrastructure remodeling, and the release of cytochrome *c* into the cell cytosol. GAS-induced macrophage apoptosis impaired the ability of the macrophages to kill GAS and to secrete cytokines, dampening activation of an immune response. GAS lacking SLO were more attenuated in a murine model of systemic infection, with less bacteria surviving in the blood and diminished mortality compared to WT GAS.

The exactly mechanism of GAS-induced apoptosis remains unknown, although we hypothesize that SLO may be forming pores in the mitochondrial membrane which elicit depolarization and the release of cytochrome *c*. Further studies are ongoing in producing a mutated SLO which is unable to form pores. We also observed that the apoptosis induced by purified SLO could be further enhanced by the presence of GAS components. Perhaps the pore-forming ability

of SLO allows bacterial components to enter the cytosol that would normally not be there and this signals the apoptotic pathway.

Although there was involvement of caspase-1 in GAS-induced macrophage apoptosis, this cell death appears to be distinct from a caspase-1-dependent inflammatory cell death termed pyroptosis (Fink and Cookson, 2005), which as been shown to be induced by other bacterial pathogens (Boise and Collins, 2001; Cookson and Brennan, 2001). Our data demonstrates that caspase-1 is playing a contributory role in the apoptosis induced by GAS, and the abrogated cytokine release does not correlate to pyroptosis. The induction of programmed cell death versus direct lysis of cells could be because one of the hallmarks of apoptosis is the non-inflammatory fashion in which cells are eliminated. Membrane-bound vesicles containing organelles are phagocytosed and eliminated with no leaking of intracellular contents and inflammatory response. It is beneficial to the bacteria to limit the activation of the immune system, allowing the infection to proceed undetected for as long as possible.

Interestingly, however, the addition of high concentrations of extracellular potassium chloride (KCl) almost completely abolished the induction of apoptosis. This points to a role for the inflammasome, independent of its induction of caspase-1. Further investigation into the specific inflammasome components involved may shed light on addition functions of this signaling complex.

Inhibition of macrophage apoptosis using a peptide inhibitor augmented their ability to kill WT GAS infections, demonstrating that the induction of apoptosis benefits the survival of the bacteria. Elucidating the specific pathway of GAS-induced leukocyte apoptosis could provide novel therapeutic targets for the treatment of GAS disease. The inhibition of apoptosis could allow for a natural enhancement of our own immune system's ability to fight the infection and could avoid some of the resistance complications that direct antimicrobial therapies have faced.

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