

Microreview

Molecular mechanisms underlying group A streptococcal pathogenesis

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Summary

Group A *Streptococcus* (GAS) is a versatile human pathogen causing diseases ranging from uncomplicated mucosal infections to life-threatening invasive disease. The development of human-relevant animal models of GAS infection and introduction of new technologies have markedly accelerated the pace of discoveries related to GAS host–pathogen interactions. For example, recently investigators have identified pili on the GAS cell surface and learned that they are key components for adherence to eukaryotic cell surfaces. Similarly, the recent development of a transgenic mouse expressing human plasminogen has resulted in new understanding of the molecular processes contributing to invasive infection. Improved understanding of the molecular mechanisms underlying the pathogenesis of GAS pharyngeal, invasive and other infections holds the promise of assisting with the development of novel preventive or therapeutic agents for this prevalent human pathogen.

Introduction

Group A *Streptococcus* (GAS) is a common human pathogen that has major healthcare and economic impact. The

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oropharynx is the most frequent GAS infection site, resulting in an estimated 500 million cases of pharyngitis per year worldwide (Carapetis *et al.*, 2005). Moreover, the presence of GAS in the oropharynx is generally considered to be a prerequisite for the development of rheumatic fever, the leading cause of preventable childhood heart disease in the developing world (Carapetis, 2007). Although less common than pharyngitis, invasive infections such as necrotizing fasciitis (colloquially termed ‘flesh-eating disease’) still number approximately 10 000 cases per year in the United States alone, causing an estimated 1500 deaths annually (O’Loughlin *et al.*, 2007).

Given its considerable importance to human health, GAS pathogenesis has long been an arena of active investigation, and many aspects have been elucidated (Cunningham, 2000). Despite these efforts, however, significant questions remain regarding how GAS causes human diseases. Importantly, no effective vaccine for GAS is available, emphasizing the need for continued efforts to better understand mechanisms of GAS infectivity. In the last several years, technological advancements including high-throughput comparative genomics and proteomics, expression microarray analysis and new animal models that better recapitulate human disease phenotypes have resulted in important insights into the molecular basis of GAS pathogenesis (Musser and DeLeo, 2005). Herein, we will review key recent advancements in GAS pathogenesis knowledge pertaining to pharyngeal and invasive infections.

Pharyngitis

Overview

The pathogenesis of GAS pharyngitis has commonly been divided into three stages: (i) adherence to the pharyngeal epithelium, (ii) acquisition of nutrients needed for proliferation and (iii) avoidance of the host immune response (Fig. 1) (Bisno *et al.*, 2003). In the last 15 years there has also been extensive study of the invasion and persistence of GAS in epithelial cells (Wang *et al.*, 2006a). In this section, we will review recent discoveries bearing on each stage of GAS pharyngitis pathogenesis

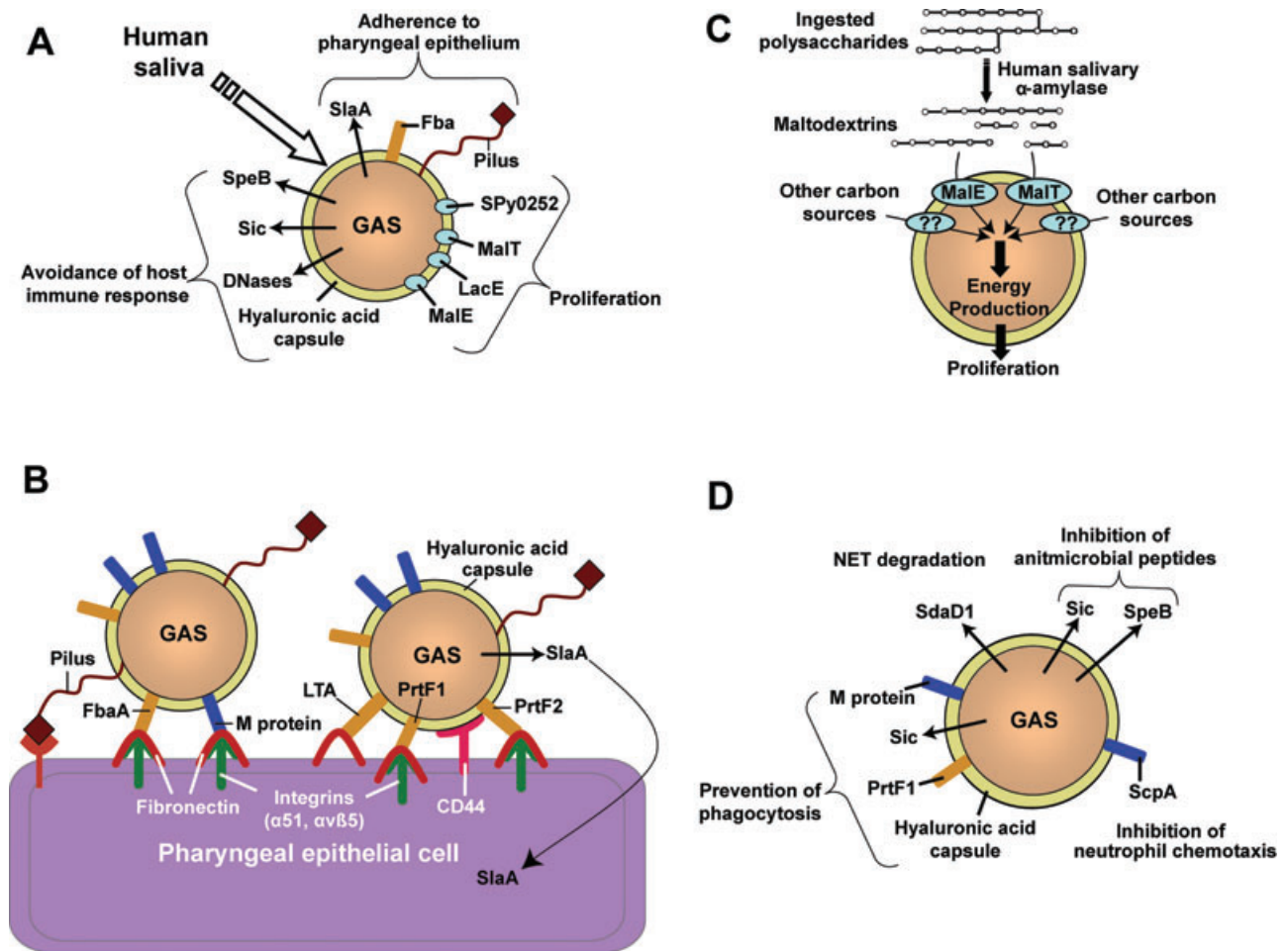


Fig. 1. GAS proteins identified as participating in pharyngeal infection divided by stages of pathogenesis. Proteins freely secreted in extracellular space are designated by arrows. Cell surface proteins are as follows: Fn binding proteins are yellow, other cell wall-linked proteins are dark blue, pilus proteins are dark brown and carbohydrate transport proteins are light blue.

A. Interaction with human saliva prepares the organism for subsequent stages in pharyngeal infection with genes encoding the indicated proteins upregulated during interaction with saliva. Genes encoding proteins noted here are upregulated during cultivation in human saliva.

B. Schematic of GAS adherence to pharyngeal epithelium. Eukaryotic proteins known to mediate GAS adherence are labelled in white. Uptake of streptococcal phospholipase A2 (SlaA) into eukaryotic cells increases GAS adherence.

C. Mechanisms of GAS nutrient acquisition needed for proliferation. The digestion of polysaccharides by human salivary α -amylase produces maltodextrins that are transported into the cells via MalE/MalT pathways. Other, yet to be identified, carbon sources likely contribute to GAS proliferation.

D. GAS proteins involved in avoidance of host immune response.

SpeB, cysteine protease; Sic, streptococcal inhibitor of complement; MalE, maltodextrin binding protein; MalT, maltodextrin phosphotransferase; LacE, lactose phosphotransferase; FbaA, FnBP A; LTA, lipoteichoic acid; PrtF1, protein F1; PrtF2, protein F2; SdaD1, streptococcal DNase I; NET, neutrophil extracellular trap.

beginning with recently developed models for investigating oropharyngeal infection.

Models for studying the pathogenesis of GAS pharyngitis

Historically, the lack of an animal model that replicates the clinical manifestations of GAS pharyngitis in humans has been a major impediment to understanding GAS pharyngeal pathogenesis. GAS can colonize the oropharynx of

baboons, but similar to mice, baboons do not develop pharyngitis following GAS inoculation (Ashbaugh *et al.*, 2000). However, unlike baboons, a human-like pharyngitis reliably occurs in cynomolgus macaques following experimental GAS inoculation (Sumby *et al.*, 2008). As a consequence, this model has been used in recent years to examine GAS genome-wide expression longitudinally during experimental pharyngitis and to discern the role of specific virulence factors in the pathogenesis of GAS pharyngitis (Table S1). The ability of the macaque model

to mimic human pharyngitis provides the opportunity to more fully elucidate the pathogenesis of GAS pharyngeal disease.

Saliva plays a central role in GAS transmission and is a key mediator of innate and acquired immunity in the human oropharynx (Amerongen and Veerman, 2002). To better study the behaviour of GAS in the human oropharynx, the interaction of GAS with human saliva *ex vivo* has been investigated (Shelburne *et al.*, 2005a; Edwards *et al.*, 2008). The finding that many genes key to GAS pharyngitis are upregulated in human saliva compared with standard laboratory medium led to the proposition that GAS–saliva interaction is the initial stage in host–pathogen interaction, thereby adding an additional step in GAS oropharyngeal pathogenesis (Fig. 1A). The relative technical ease of examining GAS behaviour in saliva *ex vivo* means that analysis of GAS–saliva interaction is a powerful tool for generating new hypotheses and understanding regarding the role of particular gene/gene products in GAS pharyngeal pathogenesis.

Epithelial cell adherence

Adherence of GAS to pharyngeal epithelial cells has been extensively investigated and much is known (Courtney *et al.*, 2002). A key recent advance has been the discovery that GAS pili-like cell surface structures are central players in pharyngeal epithelial cell adherence and biofilm formation (Fig. 1B) (Table S1). GAS pili examined to date are encoded by genes located in the so-called FCT (fibronectin, collagen-binding, T-antigen) gene region and are composed of the main pilus subunit (spy0128 in the serotype M1 strain SF370) and two ancillary proteins (Mora *et al.*, 2005). The main pilus subunit corresponds to the T antigen of the Lancefield T serotypes, one of the two major GAS classification schemes (Kang *et al.*, 2007). Assembly of GAS pili is dependent on a sortase encoded by genes in the FCT region, and at least in M3 strains, on a signal peptidase encoded immediately upstream of the T antigen (Zahner and Scott, 2007).

In addition to the pili investigations, recent studies have further elucidated the role in adherence of GAS fibronectin (Fn) binding proteins (FnBPs). Although not encoded in all GAS strains, in the strains in which it is present protein F1 (PrtF1/Sfbl) is critical for Fn-mediated GAS adherence (Hanski and Caparon, 1992). In addition to mediating adherence, a new finding is that PrtF1 inhibits C3 deposition on the GAS cell surface and confers resistance to phagocytosis (Table S1). GAS strains that lack PrtF1 may encode PrtF2, a protein critical to Fn-mediated GAS adherence in a serotype M49 strain (Table S1). Interestingly, GAS serotype M1 strains lack both PrtF1 and PrtF2 but contain FbaA, a protein that also participates in epithelial cell adherence (Table S1).

Other GAS proteins have recently been demonstrated to contribute to epithelial cell adherence. For example, the worldwide emergence of a particular clone of serotype M3 GAS has been strongly linked to the acquisition of a bacteriophage-encoded phospholipase A₂ (SlaA) that contributes to adherence to host pharyngeal epithelium (Table S1). The mechanism of action of SlaA appears to involve entry into host cells (Fig. 1B). A Δ *slaA* strain was significantly impaired in its ability to cause pharyngitis in the cynomolgus macaque, providing strong evidence of the key role of SlaA in GAS pharyngeal pathogenesis (Sitkiewicz *et al.*, 2006). Similarly, serum opacity factor also has been demonstrated to participate in Fn-mediated host cell adherence (Table S1). In summary, despite decades of investigation, the molecular basis of GAS eukaryotic cell adherence remains a very active field of investigation with new insights accumulating rapidly.

Intracellular invasion

Although GAS is predominantly an extracellular pathogen, data over the past decade have shown that the organism may invade and persist within epithelial cells (Wang *et al.*, 2006a). The exact role of this event in GAS pathogenesis is not clear. Many of the proteins involved in GAS epithelial cell invasion also participate in adherence, including FnBPs, M protein and streptococcal collagen-like protein 1 (Table S1). The eukaryotic signalling pathways mediating GAS invasion begin with bacterial binding to eukaryotic cell surface integrins, eventually resulting in actin cytoskeletal rearrangement and GAS internalization (Purushothaman *et al.*, 2003; Wang *et al.*, 2006b,c; 2007). Interaction of GAS with integrin-bound Fn results in the upregulation of transforming growth factor β 1, which in turn upregulates cell surface expression of α 5 integrin and Fn, making the cells better targets for streptococcal binding (Wang *et al.*, 2006b). The ability of GAS to invade and persist intracellularly has been associated with penicillin treatment failure and recurrent tonsillitis, but a definitive link between GAS eukaryotic cell invasion and pharyngeal pathogenesis has yet to be made (Osterlund *et al.*, 1997).

Proliferation

Development of GAS pharyngeal infection occurs following transmission of relatively small numbers of organisms from an infected or colonized host to a non-infected host. Following adhesion to the pharyngeal epithelium, GAS proliferation in the oropharynx triggers the signs and symptoms of pharyngitis. Detailed understanding of this process has been provided recently using a non-human primate model of GAS pharyngitis (Virtaneva *et al.*, 2005). Correlation of organism density with pharyngeal evaluation demonstrated that GAS proliferation preceded the

clinical development of pharyngitis, suggesting that the organism did not obtain nutrients needed for proliferation from eukaryotic cell lysis.

The concentration of glucose in fluid lining the human oropharynx is too low to support GAS growth, which means that other carbon sources are needed for proliferation to occur (Gough *et al.*, 1996). Given that carbohydrate catabolism genes are generally derepressed in the presence of their substrate, new information about the carbon source that GAS uses to proliferate in the oropharynx can be gained through analysis of gene transcript levels during experimental pharyngitis in the non-human primate. During initial GAS proliferation, genes involved in maltodextrin binding, galactose metabolism and mannose and lactose transport are upregulated (Virtaneva *et al.*, 2005). Presently, only the role of maltodextrin utilization in GAS pharyngeal infection has been examined, with most experimental analysis focusing on the cell surface maltodextrin binding lipoprotein MalE (Table S1). We have recently discovered that GAS relies on human salivary α -amylase to initiate the degradation of polysaccharides to maltodextrins that are subsequently transported to an intracellular location and enter into energy producing pathways (Fig. 1C) (Shelburne *et al.*, 2008). Salivary α -amylase levels are highly variable between individuals, suggesting that host α -amylase activity may be a factor involved in susceptibility to GAS pharyngitis (Iafrate *et al.*, 2004).

Immune system avoidance

The GAS oropharyngeal infection causes a brisk immune response in many individuals. Combating this immune response is critical to the ability of GAS to cause pharyngitis and colonize the oropharynx for prolonged periods of time (Ashbaugh *et al.*, 2000; Virtaneva *et al.*, 2005). GAS has evolved numerous mechanisms for evading the host innate immune response; the best studied include M protein and hyaluronic acid capsule (Fig. 1D) (Cunningham, 2000). The remainder of this section will focus on recent investigations examining aspects of GAS immune system avoidance participating in the pathogenesis of pharyngeal infection.

In addition to M protein and capsule, other immune modulating proteins that contribute to GAS pharyngeal infection include streptococcal C5a peptidase (ScpA), streptococcal inhibitor of complement (Sic) and secreted DNases (Table S1). ScpA is a cell surface serine protease that specifically cleaves C5a, thereby decreasing C5a-polymorphonuclear (PMN) leucocyte binding and subsequent PMN recruitment. GAS ScpA mutants are attenuated in their ability to colonize the mouse nasopharynx and active immunization with ScpA prevents nasal colonization in a mouse model (Table S1). Interestingly, the ScpA paralogue in group B *Streptococcus*, annotated

as ScpB, is a key adherence and invasion factor, and the crystal structure of ScpB has identified Fn binding domains (Brown *et al.*, 2005). The contribution of ScpA to GAS epithelial cell adherence and invasion has yet to be extensively investigated (Purushothaman *et al.*, 2004).

Sic is a secreted protein that has protean effects on host immunity in the oropharynx. The gene encoding Sic is strongly upregulated very early in the course of pharyngeal infection (Virtaneva *et al.*, 2005). In addition to inhibiting the complement membrane attack complex, Sic interferes with the function of several key aspects of pharyngeal immune defence including lysozyme, α - and β -defensins, secretory leucocyte proteinase inhibitor, monokine induced by IFN- γ /CXCL9 and the cathelicidin LL-37 (Table S1). The interaction of Sic with proteins of the innate immune system significantly decreases their bactericidal activity against GAS (Table S1). Sic also interacts with eukaryotic cytoskeletal proteins, resulting in inhibition of phagocytosis and PMN-mediated killing (Hoe *et al.*, 2002). *In vivo*, inactivation of Sic leads to decreased proliferation of GAS in human saliva and significantly impairs colonization of the mouse oropharynx (Lukomski *et al.*, 2000; Shelburne *et al.*, 2005b).

Although GAS had long been known to secrete a variety of DNases, their contribution to pathogenesis has only recently been definitively elucidated using isogenic mutant strains and the cynomolgus macaque model of pharyngitis (Table S1). Sumbly *et al.* (2005) created a serotype M1 GAS strain in which all three extracellular DNases were inactivated. Phagocytosis and killing of the triple mutant strain by PMNs occurred at a significantly higher rate compared with the parental strain. Importantly, the triple mutant strain failed to cause pharyngitis in the cynomolgus macaque, thereby clearly establishing the importance of DNases in GAS pharyngeal infection. The mutant strains were deficient in ability to degrade DNA, leading to the idea that the decreased pathogenicity was a result of an inability to degrade host-protective neutrophil extracellular traps. Buchanan *et al.* (2006) subsequently confirmed that this is the case.

Invasive infections

Overview

In this section, we will review recent discoveries bearing on the pathogen–host interactions that contribute to invasive GAS infection. Severe invasive infections such as necrotizing fasciitis are characterized by extensive tissue damage, vascular dissemination and systemic disease manifestations that result in high morbidity and mortality. Invasion may occur either by progression of an antecedent superficial bacterial infection such as pharyngitis or direct inoculation with a penetrating injury. Under either

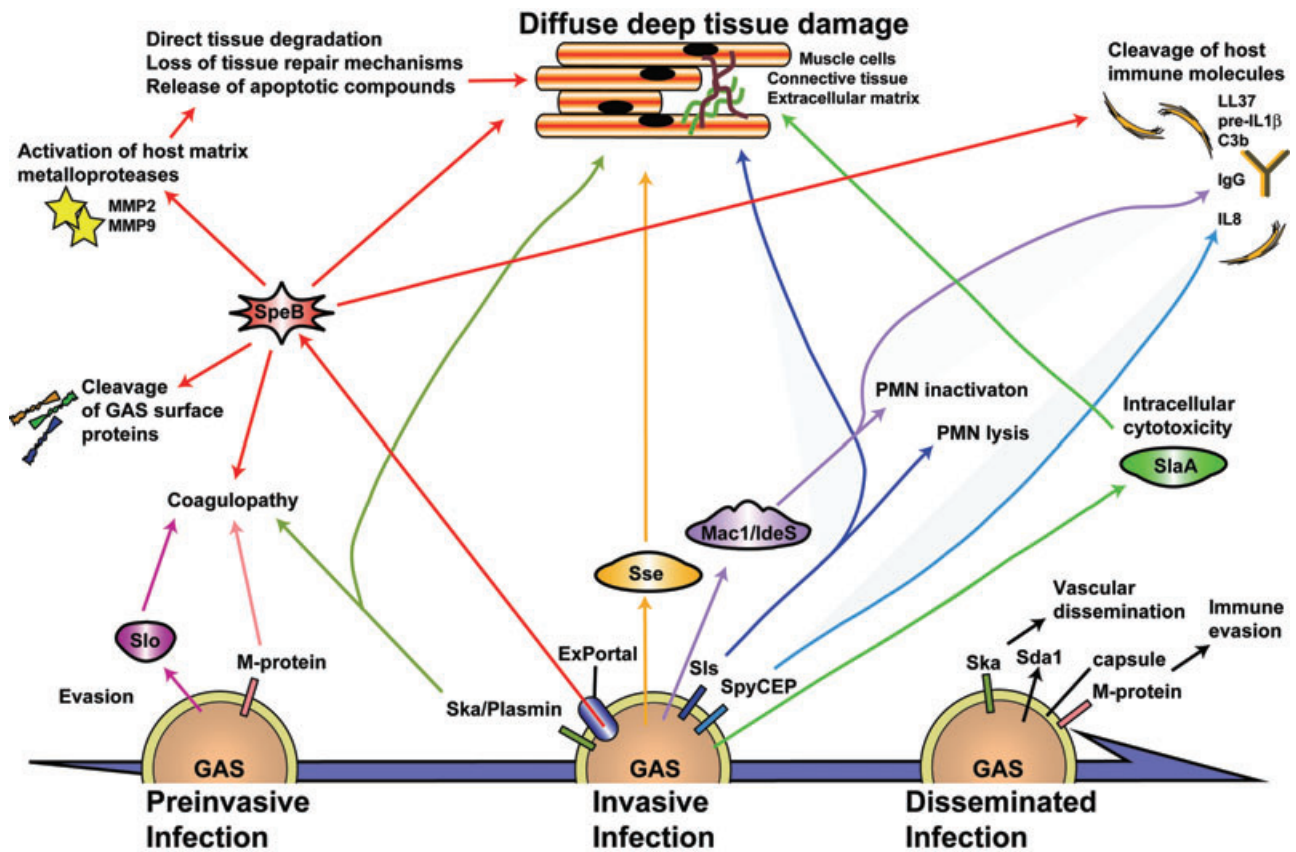


Fig. 2. Schematic representation of the complex multiple and overlapping virulence pathways implicated in invasive GAS infection. Invasive infections are characterized by severe deep tissue damage and vascular dissemination. A subset of virulence factors, including SSE, SLO and SlaA directly damage host tissue. Other virulence factors such as SLO, Mac1/IdeS and SpyCEP indirectly damage host tissue by inducing coagulopathy, inactivating PMNs and cleaving immune molecules. Finally, broad-spectrum virulence factors such as SpeB contribute to pathogenesis by several different mechanisms. The molecules shown represent only a subset of the studied GAS proteins implicated in invasive infection; additional virulence factors and pathogenesis pathways exist. Also, this figure does not depict the complexity further introduced by GAS infection stage and anatomical site-specific regulatory systems that positively or negatively regulate virulence factor expression at the transcriptional and post-translational levels.

SpeB, streptococcal pyrogenic exotoxin B, a secreted cysteine protease; SLO, streptolysin O; Ska, streptokinase; SSE, secreted streptococcal carboxylic esterase; Mac1/IdeS, immunoglobulin G degrading cysteine protease; SLS, streptolysin S; SpyCEP, *Streptococcus pyogenes* cell envelope protease; SlaA, streptococcal phospholipase A2; Sda1, streptococcal DNase I.

scenario, GAS organisms must rapidly adapt to their host environment by: (i) altering their transcriptome, (ii) expressing virulence factors that facilitate breaching local tissue barriers and enable vascular dissemination, (iii) subverting host defence and other molecules that further contribute to soft tissue damage and (iv) co-ordinating the expression of multiple regulatory systems (Fig. 2). Importantly, human genetic and acquired factors such as immunologic state also play a key role.

Models for studying the pathogenesis of GAS invasive disease

A full understanding of the marked complexity of host-pathogen interactions occurring in the heterogeneous tissue environment of invasive infections requires disease models that to a first approximation recapitulate the bio-

logical interplay between GAS and human molecules. *In vitro* and *ex vivo* assays that we and many other investigators commonly employ are useful for testing hypotheses under defined and reductive laboratory conditions, but they fail to adequately mimic the complex host-pathogen interplay occurring in the human condition. Invasive infection can be modelled in mice by inoculating bacteria into sterile sites, and virulence differences between strains can be compared by observing disease progression or other outcomes. However, GAS is a host specialist pathogen, causing natural disease only in humans. Several virulence factors thought to be crucial to invasive infection have modest or no activity against mouse molecules (Sumbly *et al.*, 2008). Thus, conclusions drawn from analysis of GAS pathogenesis based exclusively on *in vitro* and *ex vivo* assays, and mouse disease models, are inherently limiting. As such, transgenic mice

expressing human homologues of the target host proteins (Sun *et al.*, 2004) and other humanized mouse models (Svensson *et al.*, 2000) have been used. The ability of these models to better address the human disease phenotype provides a valuable tool for conducting *in vivo* molecular pathogenesis studies. As with pharyngitis, non-human primates are proving to be a reliable model for invasive GAS infection (Taylor *et al.*, 1999; Sumbly *et al.*, 2008). It is also critical, when possible, to analyse material taken from natural human infections to determine if observations made using *in vitro* and animal models are mirrored in the natural host (Sitkiewicz *et al.*, 2006).

Tissue damage

Rampant, unfettered tissue destruction and bacterial dissemination are hallmarks of severe invasive GAS disease. Extracellular proteases made by GAS have long been thought to be important contributors to invasive disease; however, many questions regarding their actual *in vivo* virulence role have gone unaddressed or incompletely answered. For decades, microbiologists utilized the genetic and biochemical techniques available to perform detailed enzymatic studies. They characterized the amino acid sequence, *in vitro* activity, target specificity and enzyme kinetics of multiple GAS molecules. Importantly, these studies laid the foundation for our current conceptualization of basic virulence mechanisms such as tissue-damaging proteases and pore-forming cytotoxins. The current challenge is to now translate these existing data to an *in vivo* context. A key recent advancement in understanding GAS virulence is the identification and enhanced understanding of a large repertoire of virulence factors. Some of this new information has been made possible by genome-wide investigative strategies, as summarized recently (Musser *et al.*, 2005). Molecular analysis of human infection isolates was crucial to these discoveries. For example, a phage-encoded GAS gene (*slaA*) for the novel extracellular secreted phospholipase A2 designated SlaA was first identified by sequencing the genome of a serotype M3 strain isolated from a patient with severe invasive infection (Beres *et al.*, 2002). Similarly, the extracellular protease SpyCEP was identified by proteome analysis of virulent GAS strains (Rodriguez-Ortega *et al.*, 2006). Given their known biological functions of cleaving fatty acid moieties and granulocyte chemotactic cytokines respectively, SlaA and SpyCEP were hypothesized to be important virulence factors for tissue invasion. Using an isogenic mutant strain, Sitkiewicz *et al.* (2006) unambiguously demonstrated that an SlaA-negative GAS strain caused significantly less mortality, tissue destruction and bacteraemia in mice, and pharyngitis in non-human primates (Table S1). Similarly, multiple investigators have independently demonstrated

altered virulence of SpyCEP-negative mutant strains (Table S1). The streptococcal secreted carboxylic esterase (designated SSE) and potent pore-forming cytotoxin streptolysin S (SLS) also have been recently shown to contribute to invasive disease pathogenesis. Importantly, Datta *et al.* (2005) reported that all nine genes in the sag locus (SagA-Sagl) are needed for SLS production (Table S1).

The finding that SlaA and Sse are secreted whereas SpyCEP and SLS are cell-localized may have important implications bearing on the mechanisms underlying their pathogenesis role. Secreted enzymes can diffusely damage host tissue, preparing it for widespread microbial invasion. In comparison, surface-retained molecules can specifically target their activity to newly encountered impediments to pathogenesis such as host immune effectors and basement membranes that must be breached prior to vascular dissemination. In essence, pathogen surface-bound molecules effectively increase the local concentration of these virulence factors. Importantly, immunization with several newly described degradative enzymes protects mice against lethal challenge, suggesting a role in GAS vaccine research. Additional pathogenesis and vaccine studies in non-human primates are needed to address the potential vaccine relevance.

Although the identification of many new GAS surface proteins has stimulated considerable interest in their potential vaccine role, one line of research provides a cautionary note. Nyberg *et al.* (2004) recently reported the unexpected result that a strain expressing the FnBP-F1 had a decreased invasion phenotype, rather than the anticipated increased virulence phenotype (Table S1). It was suggested that enhanced anchoring of GAS organisms to host cells at the initial infection site reduced the likelihood of their subsequent dissemination into tissue. The implication is significant. Once considered a vaccine candidate, this result suggested that therapeutic interventions targeting protein-F1 might actually be counterproductive. By placing selective pressure on invading GAS organisms to downregulate protein-F1, a hypervirulent strain could emerge.

Subversion of host molecules

In an apparent attempt to camouflage itself from the human immune system, GAS has evolved the highly conserved Mac1/IdeS secreted protein (Table S1). Lei *et al.* (2002) first demonstrated that Mac1, a homologue of the human leucocyte β 2-integrin, binds CD16 of host PMNs to inhibit their innate immune function. Subsequently, it was shown that Mac1 also has endopeptidase activity specific for IgG. Thus, by mimicking a host cell receptor and cleaving IgG, Mac1 potentially enhances pathogen survival in tissue. Although patients with invasive disease frequently

generate antibodies to Mac1, its actual *in vivo* role remains untested in animal models (Table S1). Similarly, the virulence effect of Mac allelic variants remains unconfirmed (Soderberg *et al.*, 2008). On a side note, Nandakumar *et al.* (2007) have recently postulated that Mac1 endopeptidase activity could be exploited to target self-reactive IgG molecules in patients with autoimmune disease. Two recently published Mac1/IdeS crystal structures may assist in the rational development of such therapeutic molecules (Table S1).

A rapidly expanding concept in GAS pathogenesis research is that invading organisms usurp host molecules to facilitate disease progression. For example, the broad-spectrum cysteine protease SpeB is well known for its ability to cleave host molecules such as Fn, vitronectin, vimentin and others, and immunologic mediators such as the antimicrobial peptide LL37 (Table S1). SpeB also activates host matrix metalloproteinases (MMPs), a finding with important pathogenesis implications. Dysregulated MMP activity may directly degrade host extracellular matrix components that serve as barriers to microbial dissemination, analogous to processes occurring in tumour cell metastasis. Also, as MMPs are responsible for the normal remodelling processes that maintain proper structure and function of injured tissue, loss of this reparative activity may intensify host tissue damage. Further, Tamura *et al.* (2004) recently demonstrated that SpeB-activated MMP2 and MMP9 stimulated the release of pro-apoptotic molecules such as TNF α and FasL from infected cells (Table S1).

Following a similar theme of subverting host molecules to generate new virulence mechanisms, invading GAS can commandeer the human plasminogen system. As an invasive infection progresses, GAS elicits factors that damage vascular endothelium, resulting in leakage of the host proenzyme plasminogen into tissue (Herwald *et al.*, 2004). GAS captures plasminogen, sequestering it with several cell wall-bound molecules such as M-protein, M-protein-related protein (Prp), glyceraldehyde-3-phosphate (GAPDH) and streptococcal enolase (SEN) (Table S1). GAS streptokinase (Ska) then converts it into plasmin, a broad-spectrum serine protease that enhances virulence. In support of this hypothesis, co-administration of purified human plasminogen significantly reduces the GAS inoculum needed to generate equivalently sized lesions in mice (Table S1). As Ska is specific for human plasminogen, early pathogenesis studies were hampered by low virulence activity with the murine homologue. The recent use of a transgenic mouse expressing human plasminogen has been crucial to expanding our understanding of this system (Sun *et al.*, 2004). In the absence of human plasminogen, *ska* expression has little virulence effect. However, humanized mice have significantly more severe disease when infected with *ska*-expressing

strains. This result highlights the need for animal models that best reproduce the human condition and emphasizes the extraordinary level of GAS host adaptation. One interesting question to be answered is whether newly discovered *ska* allelic variants that alter plasminogen activation capacity *in vitro* confer an *in vivo* consequence.

Most Ska/plasmin pathogenesis research efforts have focused on characterizing the enzymatic degradation of host tissue, but plasmin also plays a key role in coagulation. Dysregulation of the delicately maintained balance between procoagulant and thrombolytic activity may represent an additional virulence mechanism for Ska/plasmin. Coagulopathy is a well-recognized clinical feature of invasive human infections, and regional hypoxia due to vascular thrombosis is believed to significantly promote tissue damage in necrotizing fasciitis. M-protein exerts procoagulant effects by inducing tissue factor expression and activating platelets. Similarly, SpeB and streptolysin O have been linked to haemodynamic compromise via their direct and/or indirect effects on vascular endothelial cells, platelets and host-derived vasoactive compounds (Table S1). Thus, pharmaceutical manipulation of GAS host coagulation pathways may have therapeutic value. However, the Ska/plasmin complex cannot be directly regulated by any currently available coagulation inhibitors, so new agents must be developed (Parry *et al.*, 2000). The recently published crystal structure of M1-protein may be useful for undertaking this endeavour (Table S1). Another potentially promising strategy might be development of small molecules that specifically inhibit Ska or Ska production.

Localized/invasion transition

An important area of contemporary GAS pathogenesis research aims to understand how the organism transcriptome is altered at each stage of infection. Results from multiple investigators now suggest that GAS undergoes a very complex molecular transition that facilitates the progression from a localized to an invasive infection (Tart *et al.*, 2007). For example, expression microarray analysis has shown that many GAS isolates from human pharyngeal and invasive infections have distinct transcriptome profiles (Sumbly *et al.*, 2006). Importantly, when GAS isolates initially expressing a pharyngeal-type profile were passaged through mice, they acquired an invasive type expression profile and enhanced virulence phenotype. Thus, the virulence potential of a GAS strain is not determined solely by its repertoire of virulence factor genes. Rather, disease-causing capacity also is significantly influenced by differential regulation of gene expression. The CovR/CovS two-component regulatory system is key to this virulence-enhancing transcriptome switch. By negatively regulating approximately 15% of the GAS tran-

scriptome, including several virulence factors crucial for invasive disease (Graham *et al.*, 2002), CovR/CovS plays a central role in directing disease phenotype. For example, genes encoding potent virulence factors such as *speB*, *spyCEP*, *ska*, *sic* and *sdaD1* are strikingly upregulated when the CovR/CovS system is inactivated (Sumbly *et al.*, 2006). Importantly, CovR/CovS responds to a variety of environmental cues that may signal the transposition of an organism from a mucosal to an invasive site. Stimuli such as increased temperature, acidified pH and elevated magnesium are all present in necrotic tissue (Table S1). This finding provides new avenues for pathogenesis studies. Also, strategies that either alter these host environmental signals or directly block the transcriptome switch could have significant therapeutic value.

Regulatory mechanisms for virulence factor expression

Adding to the complexity of the GAS molecular pathogenesis model, cumulative data have led to a detailed understanding of virulence factor expression dynamics. For example, SpeB is expressed at very low levels during the early growth phase. An initial relative lack of SpeB allows GAS surface molecules such as M-protein and protein-F1 to interact with host cells, contributing to adhesion. Later in growth, responding to environmental stimuli such as pH and nutritional stress, *speB* expression is significantly upregulated in many strains (Chaussee *et al.*, 2001). At this point, SpeB proteolytically inactivates GAS surface molecules, a process believed to result in release of the organisms from their adhesive tethers. Concurrently, SpeB degrades host cell molecules, facilitating tissue dissemination (Table S1). It is believed that upon transitioning to a bacteremic stage, *speB* is downregulated, rejuvenating the activity of other GAS virulence factors such as M-protein and Sda1 (DNase) that are advantageous to survival in blood (Walker *et al.*, 2007). However, importantly, although *speB* may be downregulated in blood, it is clearly produced *in situ* in human tissue during necrotizing fasciitis, and thus is a critical virulence factor in severe tissue-destroying infections (Thulin *et al.*, 2006; Darenberg *et al.*, 2007; Johansson *et al.*, 2008). Furthermore, Cole *et al.* (2006) have proposed that altered expression of *speB* in a subpopulation of invading organism may contribute to GAS tissue pathology. Recently published data suggest that multiple collateral and intersecting regulatory pathways are responsible for this temporal and spatial expression pattern (Kazmi *et al.*, 2001). Other crucial GAS virulence factors are probably also regulated through similarly complex transcriptional mechanisms (Walker *et al.*, 2007). RofA-like protein 3 and streptococcal regulator of virulence are some of the most recently studied multifunctional regulators (Reid *et al.*,

2006; Kwinn *et al.*, 2007). The catabolite control protein A also plays an important virulence role in invasive disease by directly linking host metabolism and virulence factor expression (Table S1).

The GAS virulence factor regulation also occurs at the post-translational level (Woodbury and Haldenwang, 2003; Lyon and Caparon, 2004; Cole *et al.*, 2007). For example, the peptidyl-prolyl isomerase PrsA is needed for full SpeB maturation (Ma *et al.*, 2006). The ExPortal, a GAS organelle dedicated to extracellular trafficking of secreted proteins, may be particularly important to co-ordinating virulence factor production at the infection site (Table S1). However, the precise biological advantage conferred by these multiple, apparently redundant, pathways remains unknown.

Host factors

It has long been recognized that individuals infected with the same strain can develop very different infectious manifestations, but the important role of host environment, comorbidities and genetics has only recently become evident. Results from several case-control and molecular epidemiology studies have provided much new data and generated many new hypotheses.

Among paediatric patients, preceding varicella-zoster infection and age of first exposure are associated with invasive disease (Laupland *et al.*, 2000; Factor *et al.*, 2005; Yang *et al.*, 2006). Interestingly, necrotizing fasciitis is relatively uncommon among paediatric patients (Lamagni *et al.*, 2008). This suggests that age-specific host-pathogen interactions may exist for necrotizing fasciitis but not other invasive infection types. Among adult patients, intravenous drug abuse, NSAIDs, alcoholism, malignancy and diabetes predispose to invasive GAS infection. These conditions may confer a relative immunosuppression or cause other anatomical/physiological defects that lead to enhanced susceptibility. In support of this idea, hospital-based outbreaks of GAS-necrotizing fasciitis have very high mortality rates (Daneman *et al.*, 2007).

Although animal infection models assume that GAS invasion occurs either following a preceding mucosal infection or by direct inoculation, approximately one-fifth of human patients with bacteraemia have no defined primary infection, penetrating injury or risk factor (Lamagni *et al.*, 2008). In contrast, seemingly unrelated blunt trauma occurring distant from the infection site is associated with necrotizing fasciitis in humans and toxic shock in mice (Nuwayhid *et al.*, 2007; Seki *et al.*, 2008). It is believed that upregulation of vimentin expression in injured/regenerating soft tissue may be a factor, as this host molecule serves as a skeletal muscle GAS binding protein. Alternatively, any disruption of microvascular

integrity may provide a nidus for haematogenous dissemination. Thus, many important questions bearing on disease initiation remain unanswered.

Host immunological factors are being increasingly recognized for their potentially important role in conferring disease protection or predisposition. Human patients with low acute phase antibody levels to capsule and/or secreted GAS proteins are predisposed to developing severe invasive disease (Basma *et al.*, 1999; Mascini *et al.*, 2000; Akesson *et al.*, 2004). However, once GAS invades a sterile site, infection severity and antibody levels appear unrelated. Further, it may be that antibody titer is less important than antigen neutralization and leucocyte mitogenicity (Maripuu *et al.*, 2007). These findings have broad implications for vaccine development, suggesting that multivalent strategies that influence a select repertoire of host immune receptors may be needed to generate optimal protection.

It has long been suspected that host genetic factors play an important role in determining GAS disease phenotype, but knowledge of this subject is rudimentary. Consistent with this idea, certain HLA class II haplotypes may confer protection from severe systemic disease, whereas other haplotypes increase risk (Kotb *et al.*, 2002; 2003; Norrby-Teglund *et al.*, 2002). This may be related to the differential binding activities of particular HLA haplotypes for various GAS superantigens (Llewellyn, 2005). Polymorphisms in TNF microsatellite haplotypes also have been implicated (Kotb *et al.*, 2002). Thus, immunomodulatory agents may be a valuable adjuvant therapy for invasive GAS disease. In support of this hypothesis, an exuberant cytokine response is associated with increased disease severity in children (Wang *et al.*, 2008). Alternatively, monoclonal antibodies that specifically target the HLA superantigen binding region may be more efficacious. The clinical benefit of intravenous immunoglobulin preparations remains controversial (Valiquette *et al.*, 2006).

Future research

Although the aforementioned data highlight numerous important advances accomplished recently, major knowledge gaps remain. For example, the finding that GAS encodes pili that mediate adherence to pharyngeal cells is significant; however, it remains to be determined whether pili contribute to GAS pharyngitis and which specific host components they target. Similarly, although vaccination with GAS antigens can protect against disease in mice, it is not known whether a similar strategy can prevent pharyngitis and invasive disease in humans. Finally with the exception of pharyngitis, analysis of *in vivo* temporal and spatial transcriptional regulation in each stage of GAS infection is just beginning. The non-human primate pha-

ryngeal model has provided excellent data, and it is likely that additional non-human primate disease models will be equally informative.

From an invasive disease standpoint, many questions remain from a host and microbe standpoint. For instance, the critical host factors influencing development and outcome of invasive GAS infections remain unknown. The ability to readily index genetic differences in large patient populations will soon give investigators an opportunity to identify host factors underlying GAS morbidity and mortality. From the pathogen side, genome-wide investigations are clearly demonstrating that small differences in gene content, not readily detectable with standard M-protein serotyping or multi-locus sequence typing, can markedly influence virulence. Therefore, technological advancements will be crucial to future molecular pathogenesis studies. The fact that approximately 30% of the GAS genome encodes putative proteins of unknown function indicates that much remains to be learned.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Recent studies investigating the contribution of GAS factors to pharyngitis and/or invasive disease.

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