

Molecular insight into invasive group A streptococcal disease

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Abstract | *Streptococcus pyogenes* is also known as group A *Streptococcus* (GAS) and is an important human pathogen that causes considerable morbidity and mortality worldwide. The GAS serotype M1T1 clone is the most frequently isolated serotype from life-threatening invasive (at a sterile site) infections, such as streptococcal toxic shock-like syndrome and necrotizing fasciitis. Here, we describe the virulence factors and newly discovered molecular events that mediate the *in vivo* changes from non-invasive GAS serotype M1T1 to the invasive phenotype, and review the invasive-disease trigger for non-M1 GAS. Understanding the molecular basis and mechanism of initiation for streptococcal invasive disease may expedite the discovery of novel therapeutic targets for the treatment and control of severe invasive GAS diseases.

Rheumatic fever

An inflammatory disease caused by cross-reactive antibodies that are induced after a streptococcal infection.

Acute glomerulonephritis

Inflammation of the glomeruli of the kidney that follows streptococcal infection and is caused by a build-up of immune complexes.

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The Gram-positive bacterium *Streptococcus pyogenes* is also known as group A *Streptococcus* (GAS) and is an important human pathogen that is responsible for numerous diseases with diverse clinical manifestations^{1,2}. Serious postinfection immune sequelae, including rheumatic fever and acute glomerulonephritis, may also develop following repeated GAS exposure. Worldwide, GAS causes an estimated 700 million cases of mild, non-invasive infections each year, of which approximately 650,000 progress to severe invasive (at a sterile site) infections with an associated mortality of approximately 25%¹. Whereas antibiotic therapy is generally effective against non-invasive infections, severe invasive GAS infections are often more complicated to treat and may require aggressive supportive care and surgical intervention³. Unfortunately, a safe and efficacious commercial GAS vaccine has yet to be developed⁴.

GAS strains are classified by serotype based on the antigenically variable M surface protein, encoded by the *emm* gene; more than 200 *emm* sequence types have been identified⁵. Serotype M1 is among the most frequently identified serotypes from streptococcal pharyngitis⁶ and invasive diseases worldwide^{2,7}, and the resurgence of severe invasive GAS infections over the past 30 years is correlated with a single, globally disseminated GAS serotype M1T1 clone^{8,9}. Here, we define the M1T1 clone as GAS serotype M1T1 strains containing the bacteriophage-encoded virulence factors extracellular streptodornase D (Sda1) and exotoxin type A (SpeA), as described by Sumby *et al.*¹⁰. The acquisition of Sda1 and SpeA may have a key role in augmenting

dissemination and virulence potential, and distinguishes GAS serotype M1T1 from related M1 serotypes.

Invasive bacterial disease requires various virulence factors to facilitate bacterial interactions with host tissues and subversion of the host's innate immune system. The transition from localized to systemic infection by GAS serotype M1T1 is potentiated by spontaneous mutations within the genes encoding the CovRS two-component system^{11,12}, resulting in the strong transcriptional upregulation of multiple virulence-associated genes, including the operon for synthesis of the hyaluronic acid capsule and the genes encoding streptolysin O (SLO), streptococcal inhibitor of complement (SIC), NAD glycohydrolase, interleukin-8 (IL-8) protease (SpyCEP; also known as ScpC) and the DNase Sda1 (REFS 11–16). The upregulation of Sda1 expression allows GAS to escape neutrophil-mediated killing and persist at the initial site of infection through the degradation of DNA-based neutrophil extracellular traps (NETs)^{10,17,18}. Furthermore, a mutation in the *covRS* operon, which regulates around 10% of the genes in GAS, decreases the expression of streptococcal pyrogenic exotoxin B (SpeB; also known as streptopain), a broad-spectrum, secreted cysteine protease¹⁹ that cleaves several GAS virulence factors²⁰. This downregulation of SpeB protease activity prevents the degradation of the plasminogen activator streptokinase, the M1 surface protein and host plasminogen. The preservation of these proteins enables GAS serotype M1T1 to accumulate cell surface plasmin activity, which promotes invasive infection by enhancing bacterial dissemination to normally sterile sites²¹.

In this Review, we describe the clinical manifestations of invasive GAS disease, key virulence factors that are important for the progression of invasive disease and studies describing the genetic and phenotypic changes that are linked with invasive infections, and we propose a novel molecular model for the events leading to invasive-disease initiation.

Clinical manifestations and epidemiology

GAS colonizes epithelia of the oropharynx and skin, but also has the ability to penetrate epithelial surfaces to produce an array of invasive diseases, including bacteraemia, cellulitis and necrotizing fasciitis, all of which may be further complicated by the development of streptococcal toxic shock-like syndrome (STSS). Other, less common forms of invasive GAS disease include septic arthritis, puerperal sepsis, meningitis, abscess, osteomyelitis, endocarditis and peritonitis² (TABLE 1). Overall, 19% of patients with invasive GAS disease die within 7 days of infection; the development of STSS further increases the mortality rate, as 44% of patients with STSS die within a week of developing the disease²². The incidence of severe invasive GAS disease in industrialized societies (2–3 cases per 100,000 people per year) is similar in the geographically distinct regions of Europe, North America and Australia^{22–25}. An estimated 663,000 cases of invasive GAS disease occur worldwide each year, resulting in 163,000 deaths¹.

GAS virulence determinants

Invasive GAS disease requires successful colonization of the stratified squamous epithelial tissues of the skin or oropharynx. Colonization of epithelial surfaces is thought to involve an initial weak interaction with the cell surface or mucosa, mediated by lipoteichoic acid or possibly pili, to overcome the natural electrostatic repulsion between the bacterial and host cell surfaces^{26,27}. This is followed by a stronger binding through lectin–carbohydrate and/or protein–protein interactions that confer tissue specificity. Various virulence factors have been implicated in this process, some of which are strain specific and thought to confer tissue specificity, such as pili²⁸, M protein, the hyaluronic acid capsule and numerous extracellular matrix (ECM)-binding proteins². These ECM-binding proteins include members of a diverse family of fibronectin-binding proteins, such as PrtF2, SfbI (also known as protein F) and SfbII (also known as SOF), which mediate invasion of certain types of epithelial cells. However, our understanding of the precise molecular events that take place during GAS colonization of humans remains limited, in part owing to the complex nature of human epithelial surfaces and a lack of appropriate animal models for this stage of the disease. Furthermore, whereas pili of M1 GAS mediate adhesion to human tonsil epithelium and primary human keratinocytes, they are not required for adherence to immortalized epithelial cells, illustrating the limitations of some of the cell lines that are commonly used for GAS adherence and invasion models²⁸. The initial steps of colonization are not discussed further here; the reader is referred to several excellent reviews

that describe the colonization of epithelial surfaces by GAS^{26,27,29}.

After penetrating the skin, GAS encounters various barriers, including a vigorous innate immune response and different cell types to those that are encountered during the initial infection. GAS has acquired many virulence determinants that allow it to survive within this new environment (FIG. 1). GAS is a human-adapted pathogen, and although mouse infection models are extensively used for studies of GAS virulence, the high bacterial doses that are required to establish infection and the absence of key host factors often limit extrapolation to human disease.

Extracellular streptodornase D and escape from neutrophil extracellular traps. NETs are secreted by host neutrophils to facilitate the entrapment and subsequent clearance of bacteria at the initial site of infection³⁰, and they are composed of DNA, histones, granule proteases and antimicrobial peptides. GAS serotype M1T1 contains a prophage (Φ M1T1Z) that encodes Sda1, a DNase that degrades the DNA framework of NETs and thereby protects the bacteria against killing by extracellular polymorphonuclear leukocytes at the site of infection¹⁸. The presence of Φ M1T1Z (and a second prophage that encodes the superantigen SpeA, another important virulence factor) distinguishes GAS serotype M1T1 from closely related M1 serotypes. In a hypervirulent strain that was subcutaneously passaged through animals (in this strain, *covS* contains a single A insertion at position 877; TABLE 2), expression of *sda1* was upregulated fivefold, and this correlated with enhanced DNA degradation, NET clearance and increased resistance to neutrophil-mediated killing¹².

M protein. The surface-anchored M protein forms the basis for the serological differentiation of GAS strains. This protein mediates adhesion to host epithelial cells³¹ and resistance to opsonophagocytosis through the binding of fibrinogen³², complement inhibitory factor H, C4b-binding protein and immunoglobulin Fc regions³³. Furthermore, M protein increases bacterial survival in neutrophils³⁴ and NETs³⁵. Consequently, M protein is essential for full virulence in a subcutaneous mouse model of GAS invasive disease³⁶, and immunization against M protein is strongly protective in animal models³⁷. Recently, the interaction of M1 protein with collagen VI was shown to be important for subepithelial infection³⁸.

Hyaluronic acid capsule. Invasive GAS isolates are surrounded by a capsule that consists of a hyaluronic acid polysaccharide. This is a linear, high-molecular-mass polymer of glucuronic- β -1,3-*N*-acetylglucosamine that is produced by the enzymes encoded in the highly conserved *hasABC* hyaluronan synthase operon³⁹. The glucuronic- β -1,3-*N*-acetylglucosamine is nearly identical to polysaccharides that are found in the human host, facilitating GAS evasion of the host immune response². The capsule provides resistance to opsonophagocytosis^{40,41}, promotes GAS serotype M1T1 survival within

Necrotizing fasciitis
Commonly known as flesh-eating disease; an infection of the skin, causing destruction of underlying tissues and muscle.

Table 1 | **Group A Streptococcus disease symptoms and infection rates**

Disease	Symptoms	Geographical region	Rate per 100,000 per year	Mortality (%)	References
Superficial					
Pharyngitis	Sore throat, fever, tonsillar exudates and cervical adenopathy ¹³⁴	Australia	8,800	NA	135
		Australia	14,000	NA	136
		Fiji	14,700	NA	137
		India	95,000	NA	138
Impetigo	Superficial, non-follicular pustules ¹³⁹	Netherlands	2,060	NA	140*
		Fiji	90,000	NA	141
		Australia (indigenous)	23,000	NA	142
Sequelae					
Rheumatic heart disease	Mitral or aortic valve incompetence later developing into mitral stenosis ¹⁴³	Australia (non-indigenous)	40	NR	144
		India	210	2.4	145
		Australia (indigenous)	1,180	NR	144
Acute rheumatic fever	Tissue inflammation giving rise to carditis, valvulitis, arthritis, chorea, erythema marginatum and/or subcutaneous nodules ¹⁴³	Australia (non-indigenous)	1.30	NR	144
		New Zealand†	72	NR	146
		Northern Mariana Islands	85.80	NR	147
		Australia (indigenous)	224	NR	144
Acute post-streptococcal glomerulonephritis	Rapid onset of gross haematuria, oedema and hypertension, usually following an episode of GAS-mediated pharyngitis or pyoderma ¹⁴⁸	Australia (non-indigenous)	6	NR	149 [§]
		French Polynesia	18	NR	150
		Australia (indigenous)	239	NR	149 [§]
Invasive					
Streptococcal toxic shock syndrome	High fever, rapid-onset hypotension and multiple-organ failure ¹⁵¹	Europe	0.36	44	22
		Australia (non-indigenous)	0.39	23	23
		United States	0.20	35.9	24
		United Kingdom	0.27	NR	152
		Australia (indigenous)	7.43	NR	153
		New Caledonia	1.25	100	154
		Fiji	0.66	100	155
Bacteraemia	Fever, nausea and vomiting ¹⁵⁶	Europe	0.53	20	22
		Australia (non-indigenous)	0.33	NR	23
		United States	1.03	16.7	24
		United Kingdom	0.71	NR	152
		New Caledonia	5.05	7.7	154
		Fiji	2.23	NR	155
Necrotizing fasciitis	Fever accompanying rapid destruction of fascia, muscle and adjacent tissue ¹⁵⁷	Europe	0.22	32	22
		Australia (non-indigenous)	0.24	NR	23
		United States	0.25	23.7	24
		United Kingdom	0.17	NR	152
		New Caledonia	17.33	NR	154
		Fiji	0.92	NR	155
Cellulitis	Redness and inflammation of the skin, with associated pain and swelling ¹³⁹	Europe	0.89	17	22
		Australia (non-indigenous)	1.18	NR	23
		United States	1.27	8	24
		United Kingdom	1.12	NR	152
		Australia (indigenous)	37.54	NR	153
		Fiji	4.98	NR	155

Table 1 (cont.) | Group A *Streptococcus* disease symptoms and infection rates

Disease	Symptoms	Geographical region	Rate (per 100,000)	Mortality (%)	References
Septic arthritis	Fever and enlarged joints of the lower extremities, preceded by an episode of GAS-mediated pharyngitis ¹⁵⁸	Europe	0.25	9	22
		Australia (non-indigenous)	0.36	NR	23
		United States	0.27	2.7	24
		United Kingdom	0.28	NR	152
		New Caledonia	5.70	NR	154
		Fiji	1.70	NR	155
Puerperal sepsis	Fever maintained for 24 h or recurring during the period from the end of the first to the end of the tenth day after childbirth or abortion ¹⁵⁹	Europe	0.08	4	22
		Australia (non-indigenous)	0.05	NR	23
		United States	0.04	7.1	24
		United Kingdom	0.07	NR	152
		Australia (indigenous)	0.04	NR	153
		New Caledonia	2.89	NR	154
		Fiji	2.55	NR	155
Other	Meningitis, pneumonia, gynaecological infection, osteomyelitis, peritonitis or abscess	Europe	1.26	24	22
		Australia (non-indigenous)	0.98	NR	23
		United States	1.10	14	24
		New Caledonia	10.15	NR	154
		Fiji	1.74	NR	155

GAS, group A *Streptococcus*; NA, not applicable; NR, not reported. *The identity of the organism causing the disease was not reported. †No distinction between indigenous and non-indigenous subjects. ‡The original reference was not available; values are from REF. 149.

NETs through inhibition of the human cathelicidin antimicrobial peptide LL-37 (a principal constituent of extracellular traps)⁴², and is essential for full virulence in mouse subcutaneous and intraperitoneal models of invasive GAS disease^{36,41,43}. Taken together, these data suggest that a combination of GAS serotype MIT1 virulence factors, including Sda1, M1 protein and the capsule, promotes resistance to NET-based killing⁴².

Streptolysin O. SLO is a 69 kDa cholesterol-dependent cytolysin that oligomerizes to form large pores (~25–30 nm) in host cell membranes⁴⁴. The gene encoding SLO is co-transcribed with a gene that encodes NAD glycohydrolase, which is actively translocated by SLO into the cytoplasm of target cells⁴⁵. SLO has several functions in GAS pathogenesis, including the induction of apoptosis in epithelial cells⁴⁶, neutrophils and macrophages⁴⁶. SLO also facilitates the escape of GAS from the endosome-lysosome pathway following invasion of host cells^{47,48}, and the penetration of superantigens into stratified squamous cell mucosa⁴⁹. SLO mutants are attenuated in subcutaneous, intravenous and intraperitoneal mouse models of invasive GAS disease^{16,50}. Expression of SLO is higher in GAS isolates from severe invasive clinical cases than in serotype-matched non-invasive controls⁵¹.

Interleukin-8 protease. The GAS cell wall-anchored proteinase SpyCEP cleaves and inactivates the neutrophil chemoattractant IL-8 and other CXC chemokines, disrupting neutrophil recruitment to the site of infection⁵² as well as neutrophil-mediated GAS killing¹⁵. SpyCEP is essential for systemic spread of invasive GAS⁵³ after

intramuscular infection, and immunization of mice against this antigen protects the mice following intramuscular or intranasal GAS challenge⁵⁴. In addition, SpyCEP activity is correlated with the severity of invasive disease among GAS isolates, regardless of *emm* type^{51,52,55}.

Streptococcal inhibitor of complement. SIC is a highly polymorphic 31 kDa secreted protein that interferes with membrane attack complex (MAC) formation by inhibiting the interaction of the complement complex C5b67 with host cell membranes⁵⁶. As GAS is inherently resistant to complement-mediated lysis⁵⁶, the major *in vivo* effect of SIC expression is probably inhibition of other innate immune system molecules, such as human cathelicidin LL-37, α -defensins, secretory leukocyte protease inhibitor and lysozyme^{57–59}.

Immunoglobulin G endopeptidase. The immunoglobulin G (IgG)-degrading enzyme of GAS, IgG endopeptidase (IdeS; also known as Mac or MspA), is a multifunctional homologue of human leukocyte β 2 integrins that inhibits activation of neutrophils and production of reactive oxygen species by binding CD16B (also known as Fc γ RIIIB), a low-affinity Fc receptor⁶⁰. IdeS is also a cysteine protease that specifically cleaves the heavy chain of human IgG bound to the surface of GAS⁶¹, thereby preventing Fc-mediated opsonophagocytosis. Furthermore, IdeS is required for the ability of GAS to resist killing in whole human blood^{60,61}; however, the significance of this protein in animal models of invasive GAS disease remains unclear, as IdeS is not active against mouse IgG.

Cathelicidin

A mammalian cationic antimicrobial polypeptide with an important role in host innate immunity and prevention of bacterial infections.

Membrane attack complex

An assemblage of complement proteins that forms pores across cell membranes, resulting in cell death.

α -defensins

A family of mammalian cationic antimicrobial peptides that are secreted by leukocytes and inhibit the activity of serine proteases.

Lysozyme

A mammalian muramidase that catalyses the hydrolysis of bacterial cell walls.

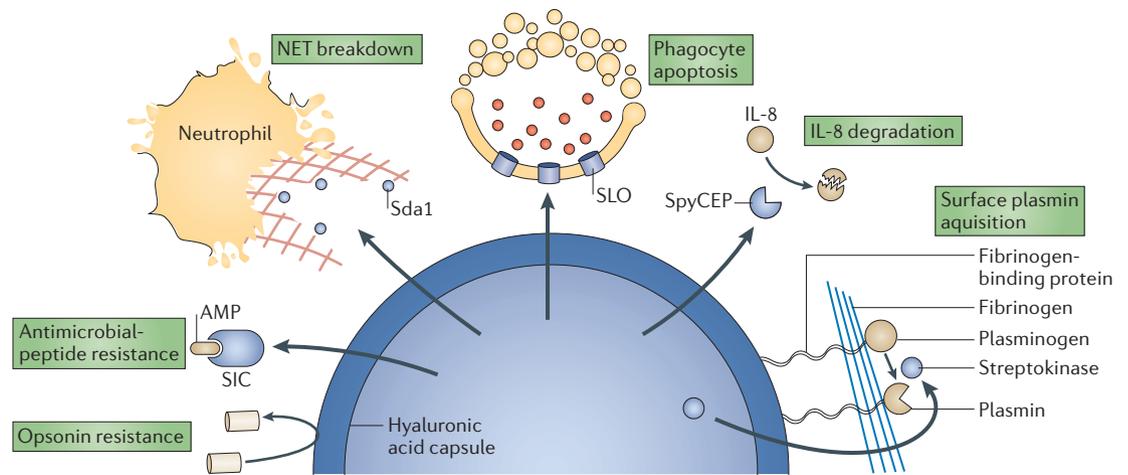


Figure 1 | The repertoire of virulence factors expressed by group A *Streptococcus* serotype M1T1 cells that disable neutrophils. Enhanced resistance to neutrophil-mediated killing is accomplished through the upregulation of multiple factors that facilitate neutrophil extracellular trap (NET) destruction (extracellular streptodornase D (Sda1)), apoptosis (streptolysin O (SLO)), interleukin-8 (IL-8) degradation (IL-8 protease (SpyCEP)) and reduced resistance to antimicrobial peptides (streptococcal inhibitor of complement (SIC) and the hyaluronic acid capsule). Fibrinogen-binding proteins help capture plasminogen, which is converted to plasmin by bacterial streptokinase. The protease activity of plasmin aids bacterial colonization of host tissues. Many of these virulence factors are upregulated in group A *Streptococcus* (GAS) strains with a mutation in the *covRS* operon, leading to hypervirulence. AMP, antimicrobial peptide.

Streptococcal secreted esterase. The virulence factor streptococcal secreted esterase (Sse) is essential for severe invasive infection and efficient systemic dissemination from the skin to the blood and organs of mice that are subcutaneously infected with a GAS serotype M1T1 isolate^{62,63}. Moreover, Sse is essential for rapid growth in human blood and serum, a characteristic that may contribute to the establishment of GAS infection by reducing GAS clearance from the host.

Streptokinase and plasmin acquisition. The streptokinase secreted by GAS converts the pro-enzyme plasminogen to plasmin^{64,65}, an activity that is similar to that of the host proteins urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). Plasmin is a broad-spectrum human serine protease that degrades blood clots and ECM components, and activates metalloproteinases⁶⁶. The presence of human plasminogen at the infection site^{67,68}, or pre-incubation in human plasma⁶⁹, enhances the virulence of streptokinase-producing GAS in mouse skin infection models, probably as the result of increased local levels of plasmin, which is proposed to have a central role in GAS pathogenesis^{65,70-73}.

Two distinct pathways, the direct pathway and the indirect pathway, mediate GAS binding to cell surface plasminogen. In the direct pathway, plasminogen receptors on the surface of the bacterium, including plasminogen-binding group A streptococcal M-like protein (PAM; also known as M53)⁷⁴, PAM-related protein (Prp)⁷⁵, streptococcal enolase (Eno; also known as SEN)^{76,77} and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; also known as Plr or SDH), bind to plasminogen directly^{78,79}. Indirect plasminogen binding occurs

through the formation of a streptokinase–plasminogen–fibrinogen trimolecular complex, which is affixed to the bacterial cell surface via plasminogen or fibrinogen receptors⁸⁰⁻⁸². Host plasmin inhibitors, including α 2-antiplasmin and α 2-macroglobulin, cannot regulate the surface-bound plasmin activity that is accumulated by GAS^{65,83}.

Invasive GAS infection is correlated with the assembly of the streptokinase–fibrinogen–plasminogen complex on the GAS cell surface^{80,81}. GAS is a highly specific human pathogen, and the streptokinase that is produced by GAS has an enhanced affinity for human plasminogen compared with its affinity for mouse plasminogen^{71,84}. Vascular leakage induced by GAS at the infection site is the proposed source of human plasminogen⁸⁵. Studies in a GAS infection model using mice that were transgenic for human plasminogen demonstrated that streptokinase and the activation of surface-acquired plasminogen to plasmin is crucial for GAS dissemination *in vivo*⁷¹, leading to the proposal that human plasmin and plasminogen are sequestered for use as virulence factors by GAS^{71,86}.

Subversion of the human plasminogen activation system by GAS serotype M1T1 plays an integral part in triggering invasive disease *in vivo*²¹. Deletion of the gene that encodes the protease SpeB in the clonal GAS serotype M1T1 isolate 5448 promotes the accumulation of human plasmin activity on the bacterial cell surface. The abrogation of SpeB protease activity at the infection site preserves streptokinase, M1 protein and host plasminogen and fibrinogen, allowing GAS serotype M1T1 to accumulate plasmin activity on the cell surface, thus promoting bacterial dissemination to normally sterile sites and helping to establish invasive infection.

Table 2 | Virulence genes of group A *Streptococcus* serotype M1T1 isolates*

Gene	Protein and function	Gene expression levels relative to wild-type controls [†]				
		<i>covRS</i> -mutant isolates from humans (ITP) [§]	<i>covRS</i> -mutant isolates from mice	<i>covS</i> 877::A mutant <i>in vitro</i>	<i>covS</i> 877::A mutant during <i>in vivo</i> colonization [#]	Δ <i>covS</i> mutant ^{**}
Antiphagocytic factors						
<i>sic</i>	Streptococcal inhibitor of complement	+	+	++	+	++
<i>scpA</i>	C5a peptidase	+	+	+	No change	+
<i>hasA</i>	Hyaluronase, involved in production of the hyaluronic acid capsule	++	++	+	+	++
<i>hasB</i>	Production of the hyaluronic acid capsule	++	++	+	+	+
<i>hasC</i>	Production of the hyaluronic acid capsule	++	++	NR	+	++
<i>ideS</i>	Immunoglobulin G endopeptidase, a CD11b homologue	++	++	No change	No change	NR
<i>spyCEP</i>	Interleukin-8 protease, a CXC chemokine protease	++	++	–	No change	+
<i>sda1</i>	Extracellular streptodornase D, a DNase	+	+	No change	+	++
<i>emm</i>	M protein	No change	No change	+	No change	++
Adhesins						
<i>fbaA</i>	A fibronectin-binding protein	+	+	+	No change	NR
<i>sclA</i>	Collagen-like surface protein	++	++	+	+	+
Toxins						
<i>sagA</i>	Streptolysin S precursor	–	–	–	–	+
<i>sagB</i>	Production of streptolysin S	–	–	NR	–	NR
<i>sagC</i>	Production of streptolysin S	–	–	NR	–	NR
<i>speA</i>	Exotoxin type A, a GAS superantigen	+	+	+	+	++
<i>speJ</i>	Exotoxin type J	+	+	No change	No change	+
<i>spyA</i>	C3 family ADP-ribosyltransferase	+	+	No change	No change	++
<i>slo</i>	Streptolysin O	++	+	No change	No change	+
<i>nga</i>	NAD glycohydrolase	++	+	No change	No change	+
Other genes						
<i>spd</i>	DNase	–	–	–	–	NR
<i>grab</i>	G protein-related α 2-macroglobulin-binding protein	–	–	–	–	NR
<i>speB</i>	Streptococcal pyrogenic exotoxin B, a cysteine protease	–	–	--	--	--
<i>ska</i>	Streptokinase	+	+	+	+	++

GAS, group A *Streptococcus*; ITP, invasive transcriptome profile; NR, not reported. *Genes that have altered transcriptional profiles following perturbation of *covRS* in GAS serotype M1T1 isolates. †++, upregulated more than tenfold; +, upregulated between twofold and tenfold; –, downregulated between twofold and tenfold; --, downregulated more than tenfold. §Data from REF. 11. Human ITP isolates are *covRS* mutants that were isolated from human invasive disease. ||Data from REF. 11. Isolates with mutations in the *covRS* locus were obtained by passage of M1 GAS strain MGAS2221 through a mouse model of invasive disease. #Data from REF. 114. *In vitro* expression data are from strains that were grown to exponential phase in broth cultures. #Data from REF. 114. *In vivo* expression data were obtained by incubating each strain in a mouse subcutaneous chamber. **Data from REF. 113. Expression data are from 18 h broth cultures of each strain.

Alterations in SpeB levels in invasive GAS

SpeB is a broad-spectrum, cell surface-associated, secreted cysteine protease that is expressed by most GAS isolates⁸⁷. The *speB* gene is highly conserved and found in >99% of GAS isolates, although there is considerable variation in SpeB expression levels among strains⁸⁸. Maximal expression occurs from late

logarithmic to stationary phase in response to nutrient availability, carbon source availability, pH and NaCl concentration^{89,90}. The protein is initially produced as a 40 kDa zymogen and undergoes conversion to the mature 28 kDa protease form by sequential autocatalytic truncation involving several intermediate forms⁹¹.

SpeB contributes to the establishment of localized skin infections²¹ and enhances GAS persistence and growth in human saliva *in vitro*⁹², but the precise role of SpeB in the pathogenesis of invasive GAS disease is not completely understood. SpeB degrades numerous host proteins, including ECM components, cytokine precursors, IgG⁹³ and antimicrobial peptides⁹⁴, to promote tissue damage and impair host immune functions². Conversely, this protease also cleaves and inactivates multiple surface-associated and extracellular GAS serotype M1T1 virulence determinants²⁰, including M1 protein⁹⁵, various superantigens^{20,96}, streptokinase⁷¹, SIC¹³ and Sda1 (REF. 20).

Loss of SpeB expression plays a key part in potentiating the transition from localized to invasive disease by sparing key GAS virulence factors from proteolytic degradation^{20,97}. Following *in vivo* passage of wild-type GAS serotype M1T1 in a mouse subcutaneous-chamber infection model, isogenic bacteria lacking SpeB activity were isolated. The secreted proteome of this passaged strain closely matched that of the isogenic Δ speB mutant that had not been passaged and that of wild-type GAS serotype M1T1 grown in the presence of cysteine protease inhibitor E-64. The levels of several virulence determinants were higher in the SpeB-negative variant than in the parental strain, including Sda1 and the superantigen SpeA²⁰. In addition, a clinical epidemiological study found an inverse correlation between SpeB expression and the disease severity of clonally related GAS serotype M1T1 isolates derived from human invasive infections of varying severity¹⁹. Specifically, SpeB levels and cysteine protease activity were significantly higher in GAS serotype M1T1 isolates from non-severe invasive infections than in isolates from severe cases, as found for STSS and necrotizing fasciitis¹⁹. Increased levels of SpeB production were correlated with the degradation of M1 protein. Other studies have also documented an inverse relationship between SpeB levels and the severity of GAS serotype M1T1-mediated disease^{93,98}. Although the downregulation of SpeB activity in GAS isolates from invasive infections is attributed predominantly to mutations in the regulator operon *covRS* (see below), mutations in the regulator gene *ropB* (also known as *rgg*) may also perturb SpeB expression^{99–101}. Deletion of *sda1* in GAS serotype M1T1 abrogated the loss of SpeB expression *in vivo* during subcutaneous mouse infection. An ancestral GAS serotype M1 isolate (SF370) that lacks Φ M1T1Z^{10,102} failed to undergo selection for the SpeB-negative *covRS*-mutant phenotype in mice that were challenged subcutaneously, when compared with selection in the wild type, indicating that Sda1 provides GAS serotype M1T1 with a selective advantage.

Disease model for invasive GAS serotype M1T1

Mutations in the genes encoding the two-component system CovRS in GAS serotype M1T1 affect the expression of virulence factors, resistance to host innate immune factors and interactions with host plasma-derived proteins, and they are important determinants of the pathogenesis of invasive GAS serotype M1T1 infection (FIGS 1, 2).

CovRS and invasive disease. Microarray analyses of a small subset of GAS serotype M1 isolates, including six isolates from cases of pharyngitis and three from invasive GAS disease, identified two distinct transcriptomes, which were designated pharyngeal transcriptome profile (PTP) and invasive transcriptome profile (ITP)¹¹. GAS isolates with the ITP profile could be recovered from mice that were subcutaneously infected with PTP GAS, and were more resistant than PTP organisms to phagocytosis and killing by human neutrophils; the transition of PTP GAS to the ITP form is thought to be stable and unidirectional. The two transcriptomes differ by approximately 10%, and the genes with differences include multiple known and putative virulence-associated genes. Specifically, transcripts were upregulated in the ITP profile for several factors that are implicated in GAS resistance to polymorphonuclear leukocytes, including the hyaluronic acid capsule, SIC, IdeS, Sda1, SpeA, streptokinase and C5a peptidase, whereas the level of *speB* mRNA was downregulated (TABLE 2). Genome-wide sequence analysis of an ITP GAS isolate recovered from a mouse infected with PTP GAS revealed that selection for a single 7 bp insertion mutation within *covS* was solely responsible for the switch from the PTP to ITP profile *in vivo*. DNA sequence analysis of 42 GAS isolates subsequently confirmed the central role of *covRS* mutations in phenotypic changes of GAS, including its pathogenesis (see [Supplementary information S1](#) (table)). Most mutations that were proved experimentally to yield the ITP profile occurred in *covS*, whereas a smaller number were in *covR* (six out of 42)¹¹; it should be noted that *covS* is approximately twice as long as *covR* and thus more likely to accumulate mutations. Many of the changes in gene expression that were induced by the *covR* mutations were similar to those induced by the *covS* mutations, though there were potentially divergent effects on a subset of genes (such as *grab* (encoding G-related α 2-macroglobulin-binding protein), *sagA* (encoding the streptolysin S precursor) and *speB*)^{11,103}. A recent study that compared the genome sequences of GAS serotype M3 isolates from human pharyngitis cases ($n=83$) and from human invasive disease ($n=215$) reported that *covS* mutations occur with a higher frequency in invasive-disease isolates than in pharyngeal isolates¹⁰⁴.

The CovRS two-component system. The *covRS* locus encodes one of the 13 two-component systems in the GAS genome and was first discovered as a regulator of capsule synthesis (and therefore initially designated *csrRS*). *covRS* is required for survival of GAS under general environmental stress conditions, such as elevated temperature, high salt concentration, low pH, presence of LL-37 and iron starvation^{105,106}, and it may have a role in the response to antibiotic stress¹⁰⁷. This system directly or indirectly regulates the expression of approximately 10% of the GAS genome, including many virulence factors¹¹, and may also be influenced by Mg²⁺ in the extracellular milieu¹⁰⁸, although the Mg²⁺ levels tested in these studies are likely to be supraphysiological. The response regulator of this system, CovR, negatively regulates the synthesis of the hyaluronic acid capsule¹⁰⁹,

Subcutaneous-chamber infection model
A disease model system that uses micropore teflon diffusion chambers which are subcutaneously implanted into mice to enable the post-infection recovery of bacteria and immune infiltrate.

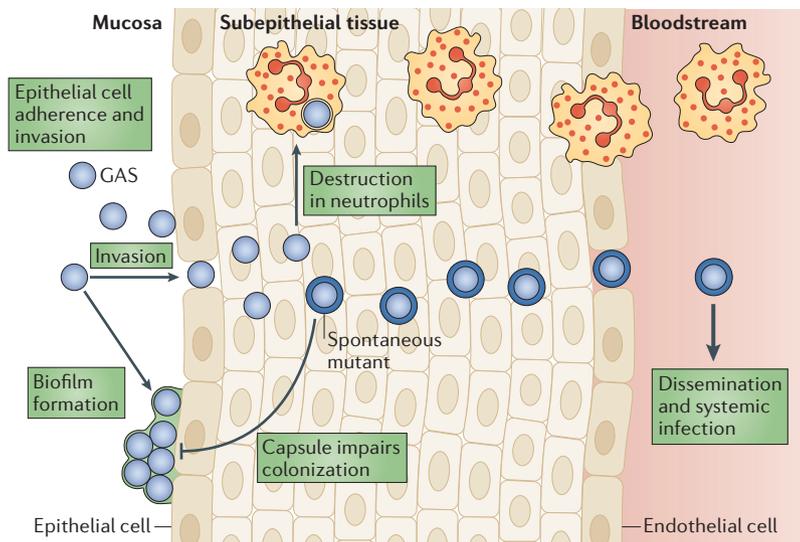


Figure 2 | Proposed model for the initiation and progression of infections with severe invasive group A *Streptococcus* serotype M1T1. Wild-type group A *Streptococcus* (GAS) serotype M1T1 adheres to and invades epithelial cells. When the bacteria gain entry to subepithelial tissue, neutrophils are recruited to the site of infection and exert selective pressure on the bacterial population for spontaneous mutations in the *covRS* operon. The resulting mutants can avoid destruction by neutrophils and, subsequently, promote tissue destruction and systemic GAS infection.

streptokinase, streptolysin S, IdeS and Sda1 (TABLE 2). CovS is a membrane-bound sensor kinase that modifies the phosphorylation state of CovR (either through phosphorylation or dephosphorylation) to reverse or enhance the repression of different CovR targets (see below)¹⁰⁵, a regulatory interaction that is essential for growth under stress conditions^{106,107}. Deletion of *covR* increases the expression of these virulence determinants and enhances GAS virulence in mouse infection models^{109,110}. CovRS positively regulates the expression of SpeB and modulates the global transcriptome profile of GAS during *ex vivo* culture in whole human blood, facilitating survival and growth¹¹¹. Several *covRS*-regulated virulence factors are potential vaccine candidates that are currently under investigation (BOX 1).

CovR retains its regulatory function in the presence or absence of a functional CovS molecule, and CovS regulates CovR to substantially enhance the repression of one subset of genes (*speA*, *hasA* (encoding hyaluronan synthase) and *ska* (encoding streptokinase)) while simultaneously reducing the repression of a second subset of genes (*speB*, *grab* and *spd3* (encoding a streptodornase))⁶² (TABLE 2). To account for this observation, it has been proposed that phosphorylated CovR strongly represses promoters of the first gene subset, whereas non-phosphorylated CovR represses the second gene subset^{62,112}. Although it is likely that CovS phosphorylates and dephosphorylates CovR, this has not been established unambiguously^{105,112}. Most of the spontaneous *covR* mutations that have been identified are non-synonymous mutations resulting in single amino acid substitutions, whereas the majority of *covS* mutations are small insertions or deletions that perturb the *covS* ORF (see Supplementary information S1 (table)).

Mutations in *covS* and certain mutations in *covR* result in downregulation of SpeB protease expression and the upregulation of several important host immune evasion proteins. Interestingly, deletion of *covS* abolishes SpeB expression¹¹³, whereas *covR*-null mutants express higher levels of SpeB than wild-type bacteria⁶². It is possible that selection at the infection site selects for *covR* mutants in which CovR retains its DNA-binding activity but is uncoupled from CovS-mediated regulation, resulting in a strain that is phenotypically identical to the *covS* mutant⁶². Thus, the *covRS* mutations that are selected for within the host rapidly change the metabolism, immune evasion functions and tissue dissemination capability of GAS and thereby promote its survival and persistence in distinct environmental niches¹¹⁴, in contrast to the wild-type phenotype, which is better adapted for the initial stages of the infection.

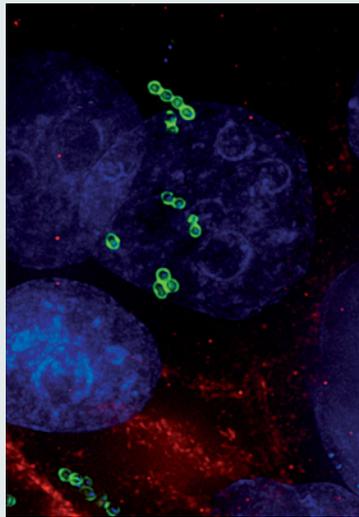
The decrease in the levels of SpeB is one of the most important changes in *covR* and *covS* mutants. SpeB is required for the establishment of localized GAS infection¹¹⁵ but also mitigates the interaction with the host plasminogen activation system by directly degrading the trimolecular streptokinase–plasminogen–fibrinogen complexes²¹. Mutations in *covR* or *covS* thus prevent the proteolysis of key human proteins (such as plasminogen and fibrinogen) and GAS surface-associated proteins (such as the plasminogen activator streptokinase and M1 protein) that are required for the activation of plasminogen to plasmin. The resulting accumulation of plasmin activity on the GAS serotype M1T1 cell surface allows the bacterium to degrade host tissue barriers and transition from the site of localized infection to the bloodstream, leading to systemic dissemination^{12,21} (FIG. 2). Interestingly, a GAS serotype M1T1 mutant strain lacking the *emm* gene did not undergo selection for the SpeB-negative *covRS* mutant phenotype in subcutaneously challenged mice⁴².

In vivo changes in *covRS* mutants. Isogenic GAS serotype M1T1 mutants harbouring precise deletion mutations in genes that are essential for virulence and/or resistance to killing by human neutrophils were used to determine which of these genes that are upregulated in *covRS* mutants are responsible for the phenotype of these mutants⁴². Importantly, the antiphagocytic M1 protein and hyaluronic acid capsule were indispensable for the *in vivo* selection of *covR* or *covS* mutations in mice, a phenomenon that was attributed to Sda1 (REF. 12). Taken together, these data suggest that the resistance to NET-based killing (a resistance that is mediated by Sda1, M1 protein and HasA) promotes the *in vivo* selection of hypervirulent SpeB-negative *covRS* mutants⁴² (FIG. 2).

SpyCEP expression is repressed by CovR, and *in vivo*-derived *covRS* mutations increase transcription of the SpyCEP-encoding gene (*cepA*; also known as *scpC*) by 100-fold⁵⁵ (TABLE 2), suggesting a central role for SpyCEP in a mouse model of invasive infection. Furthermore, SIC, which is important for growth in human blood and for virulence in mouse models of GAS infection¹³, is upregulated in a *covS* mutant^{11,113} (TABLE 2). Although expression of the *emm* gene is essentially unchanged in *covR* and *covS*

Box 1 | Group A *Streptococcus* vaccine

Group A *Streptococcus* (GAS) vaccinology aims to find a vaccine that prevents invasive GAS infections (as shown in the figure; GAS cells are labelled in green, host cell nuclei in blue and CD8, expressed from a transfected plasmid, in red) and has focused primarily on the major virulence factor, M protein. A multivalent vaccine containing amino-terminal fragments from 26 different M proteins was safe and immunogenic in Phase I clinical trials¹³³. However, this experimental vaccine does not cover all of the >150 GAS serotypes. Other vaccine candidates that are based on traditional virulence factors, including C5a peptidase, streptococcal opacity factor, fibronectin-binding proteins and pyrogenic exotoxins, have faced problems similar to those of M protein-based vaccines, including a lack of protein conservation across strains, the production of autoimmune antibodies (against group A carbohydrate) and potential toxicity as a result of the enzymatic activity of the antigen (as found for streptococcal pyrogenic exotoxin B (SpeB) and interleukin-8 protease (SpyCEP))⁴.



In recent years, reverse vaccinology techniques, aided by proteomics, whole-genome sequencing, bioinformatics and microarray technologies, have yielded a number of promising vaccine candidates⁴, including several that are regulated by the *covRS* operon (such as fibronectin-binding protein FbaA, streptococcal secreted esterase (Sse), SpyCEP and streptococcal pyrogenic exotoxin A (SpeA)) and some that are not regulated by the *covRS* operon (including C5a peptidase (ScpA), group A carbohydrate, pili and iron-binding proteins). These vaccine candidates are generally highly conserved across different serotypes and have not been observed to result in the generation of cross-reactive antibodies. Several of these vaccine candidates (namely, Sse, streptococcal immunoglobulin-binding protein 35, streptococcal protective antigen and R28) have been shown to be protective in preclinical animal studies. Thus, although these vaccine candidates are in the early stages of development, the coming years offer an exciting prospect for the development of a safe and effective GAS vaccine, and such a development would represent a major advance the field of in human health.

mutants (TABLE 2), M protein is cleaved by SpeB^{20,97} and therefore the amount of mature surface M protein is likely to be higher in *covR* and *covS* mutants than in wild-type GAS, leading to increased neutrophil resistance (FIG. 2).

Invasive disease caused by non-M1 GAS

Although GAS serotype M1T1 is clinically and epidemiologically the GAS serotype that is most frequently associated with severe invasive human disease in Western populations⁸, many non-M1 GAS serotypes can also cause invasive infections^{2,80,100,116}. Furthermore, non-M1 GAS isolates that do not produce SpeB have been recovered from human invasive infections, suggesting that *covR* and *covS* mutations may also enhance the propensity of non-M1 GAS for invasive disease. Highly encapsulated non-M1 GAS serotypes with enhanced virulence in mouse infection models have been reported. The synthesis of HasA is essential for GAS serotype M50 to colonize the upper respiratory tract of mice¹¹⁷. The mouse-passaged GAS serotype M6 isolate JRS4(HE) is a highly mucoid *covRS* mutant with elevated expression of *hasA* and *sagA* and enhanced streptolysin S activity¹¹⁸. During mid-logarithmic phase, increased amounts of capsular hyaluronic acid in this isolate reduced its

adherence to and internalization into host HEp-2 cells, in agreement with previous studies¹¹⁹. Mice that were subcutaneously challenged with this highly encapsulated GAS variant developed larger and deeper necrotic lesions than mice that were infected with the parental strain, GAS JRS4, which contains a wild-type *covRS*¹¹⁸. Furthermore, the hypervirulent phenotype could be recapitulated in the parental strain by mutating the *covRS* locus. Although secondary *covR* mutations have been reported in *covS*-mutant GAS JRS4 grown under stress conditions (such as high temperature, high osmolarity or low pH)¹⁰⁵, there is little evidence to suggest that such secondary mutations occur in GAS isolates from human invasive infections or following passage in mice (see Supplementary information S1 (table)).

GAS strain M3-f, a serotype M3 isolate that was derived from a patient with STSS, is also highly virulent in a mouse model of invasive infection and contains a non-synonymous point mutation in *covR* that reduces the ability of the encoded protein to bind promoter sequences¹²⁰. Mutation of the wild-type *covRS* genes in GAS serotype M3 increases capsule synthesis, upregulates the expression of several virulence-associated genes (including *speF* (also known as *sdaB* and mitogenic factor) and *sagA*) and enhances virulence in the mouse model. The hypervirulent *emm3*-carrying GAS isolate SSI-1 also contains a substitution mutation in *covR*¹²¹.

Mutations in *covR* and *covS* may also trigger systemic dissemination in non-M1 GAS¹²². Indeed, *covRS* mutation in non-M1 GAS is correlated with enhanced neutrophil resistance, acquisition of cell surface plasmin and virulence in a humanized transgenic-mouse model of subcutaneous infection, but the frequencies of *covR* and *covS* mutations in non-M1 GAS are significantly lower than those for GAS serotype M1T1. This reduced frequency of *covR* and *covS* mutations may explain why hypervirulent non-M1 GAS is isolated from human invasive infections less frequently than hypervirulent M1 GAS. Although the precise reason is not known for why non-M1 GAS is less able to become hypervirulent, the absence of phage-encoded *sda1* may be a contributing factor¹²².

Counterbalancing effects on colonization

Bacterial colonization of the host is a complex process that is influenced by a variety of host factors, such as the specificity and concentration of immunoglobulins in saliva, the types of mucins present and the presence or absence of normal flora on mucosal surfaces²⁶. The enhanced capsule biosynthesis that is detected in GAS mutants with an insertion in *covS* impedes bacterial adherence to human keratinocytes, most probably by masking the interaction of surface-associated GAS proteins with host cell receptors¹²³. In addition, *covS*-mutant biofilm formation and survival in whole human blood *ex vivo* is serotype or strain dependent, suggesting that *CovRS* has divergent effects on target genes in different GAS serotypes¹²³. Although *covS* mutants have enhanced virulence in mouse models of invasive GAS infection, they exhibit a lower survival in human saliva than their wild-type counterparts, suggesting that the mutants have reduced fitness in the upper respiratory tract⁶².

Furthermore, an isogenic *covS* mutant that was engendered during animal passage also has a fitness cost that counterbalances GAS serotype M1T1 hypervirulence¹²⁴. Hyperencapsulated *covS* mutants of animal-passaged GAS serotype M1T1 do not bind human epithelial cells and fibronectin as well as wild-type bacteria, and these mutants also have a diminished capacity to form biofilms, which are postulated to enhance GAS resistance to antibiotics and facilitate persistence within the human host¹²⁵. Moreover, the *covS* mutant shows reduced adherence in a mouse model of skin infection. These colonization defects were attributed to upregulation of capsule synthesis in the *covS* mutant. In summary, these data suggest that *covS* mutation confers hypervirulence *in vivo* but concurrently mitigates the colonization capacity and transmissibility of GAS serotype M1T1. The reduced fitness associated with *covR* and *covS* mutations may explain why these hypervirulent GAS serotype M1T1 mutants have not displaced the wild-type form in the human population.

***covRS* mutants from invasive human infection**

Mucoid or highly encapsulated GAS isolates have been associated with pharyngeal persistence, acute rheumatic fever and severe invasive human infections^{109,126,127}. In comparison with non-mucoid variants, the mucoid colonies are typically larger and exhibit a glistening appearance indicative of enhanced capsule production. Mutations in *covRS* have been detected in M1 GAS isolated from the human pharynx and bloodstream, as well as from the skin^{14,128} (see Supplementary information S1 (table)). SpeB-negative *covS* mutants have also been detected in M1 GAS isolates from Japanese patients with STSS and pharyngitis¹²⁹. Furthermore, *covRS* mutations have been identified in GAS isolates of various *emm* genotypes from patients with severe invasive STSS^{51,100}. Invasive STSS isolates with a *covS* mutation display enhanced resistance to killing by human neutrophils and increased virulence in the mouse infection model, and this phenotype can be attenuated by complementation with the wild-type *covS* allele⁵¹. GAS with *covR* mutations has also been identified in cases of STSS¹²⁰. The identification of *covRS* mutations in highly virulent GAS serotypes isolated from human patients suggests a pivotal role for *covRS* in severe invasive human infections^{11,120}. GAS strain H292 (*emm81*), a human blood isolate from a lethal case of bacteraemia and necrotizing fasciitis⁵², has high levels of SpyCEP activity, is hyperencapsulated and contains a single mutation in *covR* that impairs CovR binding to the *cepA* promoter⁵⁵, indicating that SpyCEP is under negative regulation by CovRS in non-M1 serotypes.

Notably, an *emm81.0*-carrying GAS isolate from the throat that had spread to the bloodstream was shown to have acquired a mutation in *covRS*¹³⁰. A pharyngeal isolate and a subsequent blood isolate recovered from the same individual after a period of 13 days are distinguished genetically by an 11 bp insertion in *covS* that results in a premature stop codon and a truncated CovS peptide. The blood isolate is deficient in SpeB protease activity, and it also has significant transcriptomic

differences to the isogenic wild-type isolate, with a transcriptomic profile similar to that reported previously for GAS serotype M1T1 (REF. 11). This blood isolate contains the phage-encoded *sda1* gene, which may give the *covRS* mutant a selective advantage in the host. This important work suggests that initial colonization is by wild-type GAS and is followed by selection for *covRS* mutations *in vivo*, leading to the progression of invasive disease in humans.

Concluding remarks

The finding that *covRS* mutations in GAS are associated with an increased capacity to initiate invasive infections has important implications for comparative analysis. Investigators should determine the *covRS* status of the standard laboratory strains under their investigation. For instance, the original M1 GAS sequence strain, SF370, possesses a wild-type *covRS* allele¹³¹; however, the GAS serotype M1T1 sequence strain, MGAS5005, bears a mutation in *covS* and displays the ITP transcriptome profile¹¹. Thus, it may be wise to avoid the use of GAS strain MGAS5005 and other strains carrying *covR* or *covS* mutations in studies of bacterial adherence and colonization, given that evidence suggests that bacteria with this ITP phenotype are not the colonizing form. Clinical studies have yet to determine whether *covRS* mutations are selected during superficial throat and skin infections, although these mutations might be predicted to occur at any site under innate immune selection. Broad epidemiological analyses of GAS isolates from non-invasive human infections have yet to be undertaken to determine whether there is an association between clinical outcome and mutations in *covRS* or other regulator genes. The mechanism by which GAS strains transit from sites of superficial infection to the focal point of necrotizing fasciitis requires further investigation. This is particularly relevant when there is no obvious trauma or skin wound that would allow easy access of GAS to deeper tissues. Although *covRS* mutants are bestowed with enhanced host tissue-degrading capabilities owing to the accretion of cell surface plasmin activity, it remains to be determined whether this phenomenon contributes to the pathology of necrotizing fasciitis. Upon entering the bloodstream, *covRS* mutants may seed damaged and/or hypoxic tissue and trigger necrotizing fasciitis, as proposed previously¹³². The origin and distribution within the GAS population of the phage that encodes Sda1 and superantigens, including SpeA and SpeC, have yet to be fully explored. Finally, consideration should be given to the *covR* and *covS* genes of GAS strains that are used in vaccination studies, given the differences between PTP and ITP phenotypes. Mouse passage to increase the virulence of GAS challenge strains in mouse models may select for the ITP form, and this may result in the identification of vaccine candidate antigens that are differently expressed in the PTP form. This is particularly important to consider because antigens that are highly expressed in the PTP form of GAS will need to be targeted if the goal of vaccine formulation is to break the colonization and transmission cycle.

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Competing interests statement

The authors declare no competing financial interests.

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