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Cellular interactions of *covR/S* mutant group A *Streptococci*

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Abstract

Group A *Streptococci* (GAS) are responsible for a wide array of non-invasive and invasive diseases and varying immune sequelae with high rates of mortality and morbidity. GAS strains with a mutation in their *covR/S* regulatory system are hypervirulent with an increased capacity for causing invasive disease. *CovR/S* mutants augment their virulence through the up-regulation of important virulence factors and target host immune surveillance primarily by inhibiting neutrophils. An in-depth understanding of the immunopathogenesis of *covR/S* mutants will facilitate the development of vaccine strategies and design. Ultimately, by targeting separate virulence mechanisms, multi-component vaccines may provide improved protective efficacy against hypervirulent GAS infections.

Keywords: *covR/S*; hypervirulent; vaccine; neutrophils

1. Epidemiology and disease spectrum

The large serotypic diversity of GAS (defined by the surface M protein) reflects the significant variation that exists within the 5' region of the *emm* gene. Large-scale epidemiological studies have highlighted a difference in circulating *emm* types within different populations [1, 2]. Generally, industrialized nations have a limited number of *emm* types, primarily 1, 3, 12, and 28, and in developing nations the *emm* gene is more diverse [3, 2].

GAS cause a broad spectrum of diseases with varying degrees of severity. Diseases caused by this pathogen range from superficial infections to life-threatening invasive diseases and post-streptococcal complications. Non-invasive GAS diseases include pharyngitis and pyoderma (Table 1). GAS is the most common bacterial cause of pharyngitis. It is estimated that over 600 million cases of GAS-related pharyngitis occur annually with the majority of cases occurring in children aged 5-15 years [4]. Non-invasive streptococcal skin infections are endemic in developing countries with prevalence rates often averaging between 1-20%. However, the prevalence of pyoderma averages between 50-90% in some Pacific Island and Indigenous Australian communities [4]. A recent systematic review estimated that the worldwide prevalence of impetigo/pyoderma was in excess of 162 million cases with most occurring in tropical, resource-poor communities [5].

It has been estimated that there are approximately 663,000 new cases of invasive GAS infection annually causing more than 160,000 deaths [4]. Invasive GAS diseases include necrotizing fasciitis (NF) and streptococcal toxic shock

syndrome (STSS) (Table 1). STSS commonly complicates other invasive GAS infections, including NF [3]. Mortality rates for NF and STSS range from 30-60% even with aggressive surgical treatment and antibiotic therapy [6].

Since the mid-1980s there has been a resurgence in invasive GAS infections dominated by *emm1*. This increase in severe GAS disease has been attributed to the acquisition of additional virulence factors (VFs) via horizontal gene transfer. The potent phage-borne DNase activity via Sda1 is believed to be the link between *emm1* and the development of invasive disease [7].

Post-streptococcal sequelae are of great importance, particularly within the indigenous populations of developed nations (including Australia and New Zealand), as well as in developing tropical settings such as India. Rheumatic fever (RF), rheumatic heart disease (RHD) and acute post-streptococcal glomerulonephritis (APSGN) comprise the major burden of streptococcal immune sequelae. Australian Aboriginal populations suffer from one of the highest documented incidence rates of RF worldwide with prevalence rates of RHD approaching 300 cases per 10,000 people in some areas [4, 8]. These non-suppurative sequelae are caused by a delayed auto-immune reaction to a previous streptococcal infection of the upper respiratory tract whereby molecular mimicry between streptococcal and human heart proteins results in auto-reactive T-cells damaging heart tissue [8]. It has been suggested that in tropical regions, untreated pyoderma can also lead to RF and RHD [9].

2. Important virulence factors and their role in GAS pathogenesis

Initiation of a GAS infection requires adherence to host epithelial or mucosal cells whilst out-competing other bacteria of the normal flora. To overcome the obstacles provided by host cells, GAS possess and selectively express surface-associated VFs necessary to establish infection. Defence against innate immunity is critical and involves adhesins such as fibronectin-binding protein, lipoteichoic acid, M-protein, and Protein F. The adherence of GAS is believed to be a two-step process with an initial weak reversible interaction mediated by lipoteichoic acid binding to fibronectin bringing bacterial and host cells into close proximity. A second stronger linkage involves multiple adhesins and relevant receptors conferring tissue specificity [10]. The M-protein is the most important adhesin used in the second linkage.

The M-protein is the primary virulence determinant of GAS. This alpha-helical coiled-coil protein is involved in numerous pathogenic mechanisms ranging from adherence to inhibition of phagocytosis. The M-protein is under strong selection pressure by the immune system and provides defence to GAS through its highly variable nature enabling new strains to evade immune recognition. The protein consists of four repeat regions that increase in sequence conservation towards the C-terminus. Conversely, the N-terminus harbors a highly immunogenic and serotype-specific hypervariable region [1].

The hyaluronic acid (HA) capsule is an important VF that enables GAS to resist phagocytosis by polymorphonuclear leukocytes [11]. The capsule is composed of HA polymers with repeating units of glucuronic acid and *N*-acetylglucosamine

that are structurally identical to the HA found in human extracellular matrix. This similarity to host tissues allows GAS to conceal its own antigens and go unrecognized by the host. The varied expression of HA at various stages of bacterial growth plays a critical role in defining the outcome of opsonophagocytosis.

Streptococcal VFs that are shed or secreted from the cell or located on bacteriophages are primarily involved in GAS dissemination through cleavage and degradation of host proteins. SpyCEP (*Streptococcus pyogenes* cell envelope protease) is present on the cell wall of GAS during bacterial growth but secreted during the stationary phase. SpyCEP has the ability to degrade the chemokine, IL-8, homologous to the murine chemokines, KC and MIP-2, thereby impairing neutrophil recruitment to the site of infection [12]. Hemolysins such as Streptolysin O (SLO) and Streptolysin S (SLS) are important secreted VF of GAS. SLO is a highly-conserved oxygen-labile, cholesterol-dependent exotoxin that exhibits potent pore-forming abilities. SLO is also able to work in tandem with the cytotoxin, NADase, by creating pores in host cellular membranes that allow translocation of NADase into cells. This results in a disruption of host co-enzyme NAD⁺ and, ultimately, cell death [13]. SLO is strongly immunogenic and the titer of anti-SLO antibodies is used as an indicator of past or current GAS infection. Conversely, SLS is an oxygen-stable and non-immunogenic cytolysin with hemolytic and cytotoxic functions. Some strains of GAS also possess streptodornase (Sda1) on a bacteriophage. This DNase augments host immunity primarily by degrading neutrophil NETs [7].

GAS also secrete a protein, streptokinase (ska), capable of activating plasminogen thus increasing accumulation of surface-associated plasmin. This allows for the degradation of host connective tissue and fibrin clots thereby facilitating invasion. A consequence of this is host tissue destruction and unregulated inflammation [reviewed in [14]]. SpeB, a broad-spectrum cysteine protease that is involved in the cleavage of several host and self-proteins such as immunoglobulins and streptokinase respectively, is instrumental in plasminogen activation due to its ability to degrade and inactivate streptokinase. SpeB is also important for initiation of infection due to its host cellular cleavage action [6]. Streptococcal pyrogenic exotoxins (SPEs) such as SpeA and SpeC are superantigens with the capacity to induce unregulated cytokine production and proliferative T-cell responses. This subsequently leads to the overstimulated host inflammatory response, characteristic of STSS disease.

3. *covR/S* regulatory system

The interactions between pathogen and host are multi-faceted and incredibly complex. This is in part due to the multitude of host immune response systems that are operating concurrently during an infection. The control of VF expression is governed by 13 separate two-component regulatory systems (TCS). These TCS integrate environmental stimuli with the metabolic state of GAS to produce a coordinated response [15]. The best-characterised GAS TCS is *covR/S*.

The *covR/S* operon is comprised of a response regulator gene (*covR*) and a sensor kinase gene (*covS*) that directly or indirectly controls approximately 15% of the streptococcal genome. In contrast to the majority of regulatory systems, *covR/S* is a negative transduction system that exerts its effects through gene repression. This system helps GAS to survive environmental stress by responding to specific signals such as increased temperature and salt concentrations, or host antimicrobial peptides indicative of environmental changes accompanying disease progression. The membrane-spanning *covS* senses particular signals within the cellular environment with ensuing activation leading to autophosphorylation. The phosphate group is then transferred to its cognate response regulator (*covR*), which resides intracellularly, resulting in differential affinities for DNA binding sites by *covR* [15, 16].

There are several VFs that are under the control of the *covR/S* system, including SpeB, SLO, SpyCEP, Ska, Sda1, and capsule [15]. A genetic mutation in *covR/S* leads to the up-regulation (or de-repression) of several VFs with the exception of SpeB, which is subsequently down-regulated. The down-regulation of SpeB is important for *covR/S* mutant disease progression as it facilitates the functionality of specific VFs that would otherwise be inactivated by its cleavage action, including Ska [reviewed in [14]]. It is due to this up-regulation of VFs that enhanced immune evasion can be achieved by *covR/S* mutants, often resulting in hypervirulence.

The sequence of events leading to and causing a mutation in *covR/S* has not been determined. The primary fitness cost of *covR/S* mutation acquisition is a

decreased ability to adhere to epithelial cells and establish colonization [17]. Therefore, it is hypothesized that a spontaneous non-reversible mutation occurs within *covR/S* after colonization enabling the up-regulation of VFs important for dissemination and the down-regulation of SpeB (Fig. 1). The resultant increased virulence, primarily in the form of neutrophil resistance, enables a survival advantage leading to invasive infections [7]. It has been hypothesized that neutrophils may actually be a requirement for *covR/S* mutation acquisition as they provide a selective pressure for the organism [18].

The accumulation of *covR/S* mutations was initially proposed to be reliant upon the possession of specific VFs by the organism. For example, it was hypothesized that the possession of the M-protein, capsule, and Sda1 were essential for the ability of GAS, generally *emm1*, to acquire *covR/S* mutations [7, 17]. However, studies have since shown that possession of Sda1 is not a prerequisite for *covR/S* mutation, and mutants are not limited to *emm1*, but cellular interactions with specific VFs do play an important role in *covR/S* mutant pathogenesis [19].

4. Cellular interactions during hypervirulent GAS infection

The establishment of a GAS infection requires efficient adaptation to the surrounding environment. In general, it is the nature of the interactions between GAS and host cells that determines the success of infection.

At the initial stages of GAS infection, adherence and colonization to host epithelial cells are critical. It is advantageous for GAS to come into close contact

with host cells during the establishment of infection. However, a recent study observed that an M1T1 *covR/S* mutant exhibited reduced epithelial cell binding and a decreased capacity to bind fibronectin *in vitro*. Furthermore, the same *covR/S* mutant displayed significantly less ability to adhere to murine skin *in vivo*, signifying a reduced ability to establish GAS infection [17]. This was shown to be heavily dependent on up-regulation of the capsule-encoding genes. Similar studies have shown that *covR/S* wild-type GAS out-compete *covR/S* mutant GAS in human saliva [16]. These close interactions with host cells that are imperative for initiation of infection are disadvantageous for systemic infections whereby host cells are actively seeking to kill GAS. Therefore, the up-regulation of significant VFs by *covR/S* mutants appears to come at a fitness cost. Nevertheless, once these *covR/S* mutant strains become systemic within the host, the altered virulence profile hindering them during colonization now provides a selective advantage.

A primary function of *covR/S* mutant GAS is aimed at neutrophil inhibition. Several of the VFs that are up-regulated upon mutation target the innate defense provided by neutrophils. For example: increased SpyCEP expression results in increased IL-8 cleavage and a subsequent reduction in neutrophil chemotaxis [20]; up-regulated SLO enables an increased ability to induce pores in neutrophil membranes leading to cellular apoptosis/necrosis [21, 13]; mucoid strains are able to avoid phagocytosis by neutrophils more effectively [11]; and for GAS strains that possess Sda1, an increase in neutrophil NET degradation ensues [7]. Neutrophils are both an essential component of the host immune system and a primary target for evasion by *covR/S* mutants during infection.

238

239 The interactions of macrophages with GAS show some commonality with
240 neutrophil-mediated innate defense mechanisms. In a functional immune
241 response, macrophages are able to phagocytose and kill GAS via intracellular
242 enzymatic degradation. However, recent studies have highlighted the ability of
243 some GAS strains to survive intracellularly within the macrophage phagocytic
244 vacuole as a means of host immune evasion and dissemination [22, 23]. The
245 cytotoxic activity of SLO has been implicated in this intracellular survival
246 mechanism. The expression of SLO is key to enabling immune evasion of both
247 neutrophils and macrophages, and has shown to be both necessary and sufficient
248 for GAS-induced macrophage apoptosis [21]. Not only does the intracellular
249 survival of GAS provide a means of dissemination and evasion of
250 immunosurveillance, but it also enables phenotypic switching of GAS to more
251 virulent phenotypes characterized by increased capsule production and an up-
252 regulation of the *covR/S* TCS over time [22, 23]. Recent studies from our
253 laboratory have also demonstrated that SLO is an essential VF for *covR/S* mutant
254 hypervirulence leading to evasion of host immunity during superficial skin
255 infection (unpublished data).

256

257 **5. Combining knowledge for vaccine advancement**

258 The high morbidity and mortality associated with GAS infections underscores
259 the need for an effective GAS vaccine. There are currently no licensed GAS
260 vaccines; however, some candidates have shown promise in preclinical studies
261 and have progressed to human clinical trials [reviewed in [24]]. Broadening our

understanding of cellular interactions between host cells and GAS has led to the development of multi-component vaccines. By targeting separate virulence mechanisms, combination vaccines are better suited to protect against hypervirulent GAS.

A recent redesign of an M-protein-derived vaccine candidate has led to increased protective efficacy against hypervirulent GAS in a murine model of pyoderma. The J8-DT vaccine candidate utilised a highly-conserved M-protein cryptic epitope (J8) to induce opsonic antibodies capable of recognizing multiple GAS strains. Recent studies have shown that this protection is mediated by, and significantly reliant upon, efficient neutrophil chemotaxis and bacterial killing [25]. Protection afforded by J8-DT against hypervirulent *covR/S* mutants was significantly reduced. The supposition that *covR/S* mutants significantly up-regulate SpyCEP to impede neutrophil chemotaxis was the rationale behind the development of a combination vaccine. The addition of a 20-mer peptide (S2) from SpyCEP to J8-DT provided a synergistic protective response better equipped to afford protection against hypervirulent *covR/S* mutant infections [26].

Another combinatorial approach was taken whereby several peptides representing important GAS VFs were combined to induce protective antibodies against multiple antigens [27]. This candidate incorporated inactivated GAS peptides in various combinations, including SLO, SpyCEP, C5a peptidase, J8, and the group A carbohydrate lacking the *N-acetylglucosamine* side chain. These vaccine candidates induced high-titer antigen-specific antibody responses with

bactericidal activity in murine sera. Protection against cutaneous infection in mice was observed for each combination vaccine candidate.

Putative GAS proteins for use within combination vaccines have also been identified based upon high-throughput techniques including mass spectrometry rather than traditional *in vivo* animal models. Combination vaccine candidates identified using this proteomic/immunomic approach induced bactericidal antibodies and cross-protection against multiple GAS strains [28].

6. Conclusion

Hypervirulent *covR/S* mutant GAS display altered cellular interactions during infection. Therefore, gaining a more thorough understanding of the cellular interactions occurring during these infections not only enables insights into the complex nature of GAS pathogenesis, but it also allows for improved strategies for vaccine design. The ability to target distinct immunological aspects of GAS pathogenesis, and combine them in a synergistic manner, will enable a broader coverage of protection against hypervirulent GAS strains of various *emm* types.

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Fig 1. Proposed model for *covR/S* mutation during group A streptococcal skin infection. A spontaneous non-reversible mutation within the *covR/S* regulatory system of GAS causes the de-repression of several important virulence factors, including Sda 1 [7], hyaluronic acid capsule [17], SpyCEP [20], SLO [21] and streptokinase [15], and the down-regulation of SpeB [7]. This altered virulence profile results in hypervirulence and invasive disease in the host.

Table 1. Common diseases caused by GAS.

Disease	Infection type	Primary tissues affected	Symptoms
Pharyngitis	Non-invasive	Throat	Sore throat, tonsillar exudate, fever, headache, body aches
Pyoderma	Non-invasive	Skin	Purulent crusted skin lesions
Necrotizing fasciitis	Severe/invasive	Skin, blood	Severe tissue damage, necrosis, septic shock, nausea, fever
Streptococcal toxic shock syndrome	Severe/invasive	Skin, blood	Widespread organ failure, septic shock, nausea, fever, hypotension
Rheumatic fever	Post-streptococcal	Throat, skin, joints, brain	Painful joints, fever, nodules under the skin, rapid movements
Rheumatic heart disease	Post-streptococcal	Heart	Heart murmur, chest pain, breathlessness, enlarged heart, heart valve damage
Acute post-streptococcal glomerulonephritis	Post-streptococcal	Kidneys	Hematuria, proteinuria, facial edema, hypertension