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# The Emergence of Hypervirulent M1T1 Clone of Group A *Streptococcus* via Genetic Recombination and Host Selection

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**Abstract**

*Streptococcus pyogenes* (Group A *Streptococcus*, GAS) is a strictly human bacterial pathogen. Since the mid-1980s, GAS M1T1 clone has been the most prevalent and globally disseminated serotype and is the culprit causing invasive and severe streptococcal infections, urging a better understanding of the emergence of hypervirulent M1T1 clone from an evolutionary perspective. This review highlights the molecular and evolutionary events leading to pandemic M1T1 strains, and discusses the pressure driving the genetic acquisition of novel virulence genes and the selection of hypervirulent isolates in host. By understanding the evolutionary selection and pressures that select and shape the pandemic M1T1 clone, we could potentially develop new therapeutic strategies to tackle challenges when dealing with the globally disseminated M1T1 GAS clone.

## Introduction

*Streptococcus pyogenes* is a Gram-positive human-restricted pathogen causing β-hemolysis of red blood cells and also known as Group A *Streptococcus* (GAS) due to the Lancefield group A antigen expressed on its surface (Lancefield, 1933). GAS primarily colonizes the nasopharynx and the skin of healthy people including children, without causing symptoms of diseases (Kline and Runge, 1994; Shaikh et al., 2010). However, when epithelial barrier damage occurs or our immunity becomes weakened, GAS is able to pass through the nasopharynx or skin barrier, and causes a wide variety of infections in human (Carapetis et al., 2005; Ralph and Carapetis, 2013). These infections result in mild diseases such as pharyngitis (strep throat) and impetigo (skin infection), and severe and life-threatening diseases such as necrotizing fasciitis (the flesh-eating disease) and streptococcal toxic shock syndrome (Carapetis et al., 2005; Ralph and Carapetis, 2013). Moreover, people infected by GAS have a risk to develop autoimmune complications, *e.g.*, rheumatic fever and acute postinfectious glomerulonephritis (Carapetis et al., 2005; Ralph and Carapetis, 2013). It is estimated that 700 million GAS infections occur worldwide each year, of which 2.9% of cases are severe and invasive, leading to more than 500,000 deaths per year (Carapetis et al., 2005).

In concert with its strikingly capacity to cause diseases, GAS has been well equipped to colonize various hostile niches and to evade host protective immunity (Cole et al., 2011; Lappin and Ferguson, 2009; Olsen and Musser, 2010). In addition to the nasopharynx and the skin, GAS has been identified as an infectious agent in muscle, kidney, lung, heart, and brain (Cole et al., 2011; Lappin and Ferguson, 2009; Olsen and Musser, 2010). To colonize and invade these tissues and organs, GAS needs to overcome tissue barriers and the immune defense elicited in various niches. To cause necrotizing fasciitis, GAS exhibits sophisticated virulence mechanisms that initiate host cell adhesion, soft tissue entry, immune evasion and tissue destruction (Olsen and Musser, 2010). In patients with streptococcal toxic shock syndrome, GAS releases superantigens to bypass normal antigen processing, causing a cytokine storm that induces an acute multi-organ failure (Lappin and Ferguson, 2009).

The most known virulence factor of GAS is the M protein, which interacts with its host ligands, *e.g.*, complement, immunoglobulins and fibrinogen, playing relevant immunomodulatory functions (Smeesters et al., 2010). As a highly variable molecule, M protein together with surface T antigen are employed to classify GAS serotypes (Lancefield, 1962). Among the >200 GAS known serotypes, M1 is the most frequently identified serotype associated with severe and invasive infections (Steer et al., 2009; Sumby et al., 2005b). For example, M1 isolates share 18.3% of all GAS isolates worldwide, and particularly dominate in high-income countries, Asia, and Latin America (Steer et al., 2009). However, since the mid-1980s, the subclone of M1 GAS, M1T1, has been rising to be the most prevalent and globally disseminated serotype and deserved greater attention (Aziz and Kotb, 2008; Maamary et al., 2012; Nasser et al., 2014; Sumby et al., 2005b).

In recent years, omics studies have provided more evolutionary and molecular based evidence for the success of M1T1 clone. This clone evolves through a series of genetic recombination events that acquire genetic regions encoding significant streptococcal virulence factors (Aziz and Kotb, 2008; Nasser et al., 2014; Steer et al., 2009; Sumby et al., 2005b). Furthermore, the M1T1 clone frequently underdoes spontaneous mutations in its major regulatory genes, leading to the emergence of hypervirulent isolates with distinctive switching of phenotypes (Aziz et al., 2004b; Ikebe et al., 2010). This review aims to outline the molecular and evolutionary events leading to pandemic M1T1 strains, and to pinpoint the mechanisms governing the phenotypic shift of M1T1 GAS clone, which underlie the selection of hypervirulent isolates in the host.

## I. Genetic acquisition of novel virulence genes determines the success of M1T1 GAS clone

The past 30 years have witnessed the emergence of the highly successful M1T1 GAS clone. Comparing to other non-M1 and noninvasive M1 types of GAS, M1T1 clone shows more advantages in host adaptation, survival, persistence and dissemination (Maamary et al., 2012; Nasser et al., 2014). These M1T1 clones

including widely studied MGAS2221 and strain 5448, are more virulent in mouse and primate model of pharyngitis and invasive infections (Aziz et al., 2005; Sumby et al., 2005b). How M1T1 clone developed such advantages remained a mystery until high-throughput sequencing technologies were adopted to comparatively analyze genetic compositions of pandemic M1T1 clonal isolates. The comparative genetic analysis reveals that the emergence of M1T1 clone is attributed to two genetic recombination events that acquire regions encoding significant streptococcal virulence factors (Aziz and Kotb, 2008; Maamary et al., 2012; Nasser et al., 2014; Sumby et al., 2005b). The acquisition of these genes in a stepwise fashion confer the versatile capacity of M1T1 GAS in host adaptation and invasiveness through synergistic effects.

*1. The first event: acquisition of bacteriophage-encoded DNase Sda1 and superantigen SpeA2*

Genomic analysis demonstrates the presence of novel bacteriophages or prophages encoding the extracellular deoxyribonuclease (Sda1, also called SdaD2) and the streptococcal pyrogenic exotoxin A (SpeA2) in the genome of M1T1 clones (Aziz et al., 2005; Banks et al., 2004). Although GAS harbors remarkable bacteriophages, which account for more than 10% of the total genome in certain M serotypes (Beres et al., 2002; Ferretti et al., 2001), the novel bacteriophages encoding DNase Sda1 and superantigen SpeA2 are exclusively found in the invasive strains, particularly in the epidemic M1T1 clone. Early at 1996, Cleary et al. observed that the pandemic M1T1 subclone differs from its closely related M1 subtypes by nearly 70 kb of two prophage DNA (Cleary et al., 1998). Through analysis of a M1T1 genome, Aziz et al. reported that these unique prophages encoding Sda1 and SpeA2 were introduced horizontally into the M1T1 global strain, and proposed that such a genetic recombination could contribute to the emergence and diversification of the M1T1 subclone (Aziz et al., 2005; Aziz and Kotb, 2008). The precise genetic recombination was later elucidated by Maamary and colleagues (Maamary et al., 2012). They showed that the acquisition of Sda1 occurred relatively early in the molecular evolutionary history of M1T1 strains, followed by acquisition of the superantigen SpeA2 (Maamary et

al., 2012; Nasser et al., 2014). Notably, the acquisition of Sda1 and SpeA2 does not necessarily improve the ability of M1 GAS to survive the killing of human neutrophils *in vitro* (Maamary et al., 2012). Instead, Sda1 and SpeA2 increase GAS virulence in the subcutaneous infection model of humanized plasminogen transgenic *AlbPLG1* mice (Maamary et al., 2012). However, M1 strain SF370, which contains four different bacteriophages encoding several superantigen-like proteins but not Sda1 and SpeA2, is less virulent and noninvasive, and genetically viewed as a pre-epidemic M1 serotype (Ferretti et al., 2001). To some extent, the acquisition of Sda1 and SpeA2 can be defined as evolutionary markers that distinguish epidemic M1T1 from non-invasive M1T1 and M1 serotypes.

1.1 DNase Sda1 and its role in GAS virulence

M1T1 GAS clone secrets several distinct DNases. Of these DNases, Sda1, was originally discovered by Aziz et al. in 2004 in culture supernatant of a M1T1 clinical isolate. Sda1 was determined to be bacteriophage-encoded (Aziz et al., 2004a) and found to be highly similar in amino-acid composition to streptodornase D from M49 GAS. Recombinant Sda1 can degrade both streptococcal and mammalian DNA, and its nuclease activity depends on the availability of divalent metal ions such as $Ca^{++}$ and $Mg^{++}$ (Aziz et al., 2004a). The crystal structure of Sda1 has been recently solved, revealing surface residues of Sda1 such as Arg12 and Asn211 involved in DNA binding and catalysis (Moon et al., 2016). It has been shown that Sda1 expression is regulated by the peroxide responsive regulator PerR under oxidative stress (Wang et al., 2013). However, the cysteine proteinase SpeB secreted by GAS seems to play a key role in controlling Sda1 expression by degrading Sda1 (Nelson et al., 2011; Walker et al., 2007).

Sda1 is a virulence factor of GAS with multi-faceted functions. Sumby et al. demonstrated that Sda1 alone is sufficient to induce wild-type levels of skin lesions in mouse model of skin infection, indicating that Sda1 but not other DNases (namely Spd and Spd3) is the major DNase that contributes to virulence of MGAS5005 in mouse models (Sumby et al., 2005a). Furthermore, the virulence of Sda1 was extensively characterized by Buchanan et al. using M1T1 strain 5448

(Buchanan et al., 2006). They found that Sda1 promotes GAS neutrophil resistance and virulence through degrading neutrophil extracellular traps (NETs), which is a significant innate immune structure composed of chromatin and granule proteins (Brinkmann et al., 2004; Buchanan et al., 2006). A recent study further suggested that Sda1 could also cleave bacterial DNA to prevent TLR9-mediated recognition of GAS by host innate immune cells, which damped the secretion of inflammatory cytokines, resulting in GAS survival in a murine model of necrotizing fasciitis (Uchiyama et al., 2012).

DNase as a bacterial virulence factor is not limited to GAS alone. Sda1 homologues can be found in various virulent pathogens, such as *Plasmodium falciparum*, *Staphylococcus aureus* and *Streptococcus pneumoniae* (Aziz et al., 2004a; Beiter et al., 2006; Thammavongsa et al., 2013). These DNases share the similar function in degrading NETs, suggesting their potential as a therapeutic target. Inhibition of DNase activity or development of therapeutic antibodies neutralizing the activity of these enzymes may facilitate the resolution of pathogenic infections. As an example, mice immunized with recombinant *Plasmodium* DNase exhibit increased immunity against lethal challenge of *P. falciparum* (Chang et al., 2016).

### 1.2 The superantigen SpeA2 – a potent scarlet fever toxin

GAS produces numerous pyrogenic toxins called superantigens. Unlike the normal antigens that are presented within the major histocompatibility complex class II (MHC II) of antigen-presenting cells and the T cell receptor (TCR), superantigens are capable to crosslink MHC II and TCR outside the peptide-binding groove, causing non-specific activation of T cells and an immense cytokine release (Fraser and Proft, 2008; Li et al., 1999). Therefore, superantigens have been implicated as the major culprit of acute infections such as sepsis, necrotizing fasciitis, and toxic shock syndrome (Norrby-Teglund et al., 2001; Proft et al., 2003; Salgado-Pabon et al., 2013; Sriskandan et al., 1996). As a representative of superantigens, SpeA has been known as a scarlet fever toxin, preferentially stimulating human T cells bearing specific TCR β-chain variable

domains (Abe et al., 1991; Braun et al., 1993; Leonard et al., 1991). Accordingly, SpeA has been shown enhancing acute nasopharyngeal infection by GAS in mice expressing human MHC II, while vaccination with inactive SpeA or SpeA devoid of MHC II binding protected mice from GAS infection (Kasper et al., 2014; Zeppa et al., 2017).

GAS encodes four SpeA variants, namely SpeA1 through SpeA4. SpeA2 differs from SpeA1 by a single amino acid substitution at position 80 from glycine to serine, which is close to the zinc-binding site in SpeA for recognition of the MHC II molecules (Earhart et al., 2000; Kline and Collins, 1996, 1997; Nelson et al., 1991; Papageorgiou et al., 1999). Importantly, all contemporary M1 isolates exclusively harbor the SpeA2 allele (Musser et al., 1991; Musser et al., 1993). In fact, a more recent research performed by Nasser et al., after analysis of genome sequences from 3,615 M1 strains, indicate that SpeA2 variant subsequently evolved from the old SpeA1 variant via a single-nucleotide change leading to glycine-to-serine substitution (Nasser et al., 2014). Although only one single amino acid difference from SpeA1, recombinant SpeA2 has a higher binding affinity for the MHC II molecules (Abe et al., 1991; Kline and Collins, 1996, 1997; Papageorgiou et al., 1999). This increased binding affinity may lead to more severe effects in the host, and could explain why SpeA2 has been detected in a majority of M1T1 GAS strains isolated from patients with streptococcal toxic shock syndrome (Musser et al., 1991; Musser et al., 1993).

SpeA2 expression is controlled by multiple factors, either from GAS or from host. Kazmi et al. initially failed to induce SpeA expression in invasive M1T1 isolates at varying culture conditions, whereas detected SpeA2 production from mouse-passaged isolates (Kazmi et al., 2001). The expression of SpeA2 was shown to correlate reciprocally and temporally to the downregulation of SpeB, and it was regulated negatively by the global regulator, the control of virulence two-component system CovRS (also known as CsrRS) in M1T1 (Kazmi et al., 2001; Sumby et al., 2006). Interestingly, SpeA2 expression can be induced in cocultures with human peripheral blood mononuclear cells *ex vivo* (Kansal et al., 2005). The

host signals, such as transferrin and lactoferrin, play a role in regulation of SpeA2 expression, and may be implicated in GAS invasive infections (Kansal et al., 2005).

*2. The second event: replacement through recombination of streptolysin O and NAD$^+$-glycohydrolase*

The second genetic recombination event occuring is that M1T1 clone acquires a 36-kb chromosomal region, which encodes streptolysin O (SLO) and NAD$^+$-glycohydrolase (NADase, also called *Streptococcus pyogenes* NAD-glycohydrolase, SPN). SLO is an oxygen-labile streptococcal hemolytic exotoxin that belongs to a large family of cholesterol-dependent cytolysins (Tweten, 2005), while NADase is an enzyme that cleaves β-NAD$^+$ to produce ADP-ribose and nicotinamide (Ghosh et al., 2010; Grushoff et al., 1975). SLO and NADase, known as well-characterized secreted toxins of GAS, have been widely found in isolates from patients with toxic shock syndrome and other severe infections. For instance, Muller-Alouf et al. reported all 212 isolates from patients with severe invasive GAS infections secreted SLO (Muller-Alouf et al., 1997). Furthermore, a strong association has been proposed between isolates causing severe invasive GAS infections and the production of NADase, and all M1 strains isolated after 1988 were positive for NADase (Stevens et al., 2000; Tatsuno et al., 2007). Compared to bacteriophage-encoded Sda1 and SpeA2, which are exclusively found in the epidemic M1T1 clones, SLO and NADase are chromosome-encoded and present in many invasive M types of GAS such as M1, M3 and M12 (Ikebe et al., 2010; Muller-Alouf et al., 1997). However, by analyzing single-nucleotide polymorphism of contemporary GAS isolates, Sumby et al. identified a considerable difference between genomes of pre-epidemic M1 strain SF370 and M1T1 isolate MGAS5005 (Sumby et al., 2005b). The latter contains a ~36 kb chromosomal region encoding SLO and NADase, which was suggested to be acquired from a M12 GAS strain by horizontal gene transfer and recombinational replacement (Sumby et al., 2005b). It is believed that acquisition of this 36-kb region together with the integration of bacteriophage-encoded Sda1 and SpeA2, cooperatively leads to the emergence and rapid global spread of the

contemporary epidemic M1T1 clone (Nasser et al., 2014; Wilkening and Federle, 2017; Zhu et al., 2015).

2.1 SLO – the potent pore-forming toxin

Nearly all GAS clinical isolates possess a highly conserved *slo* gene encoding SLO, which is detectable during exponential and early stationary growth phases of GAS isolates in the culture medium (Alouf, 1980). Moreover, SLO expression is highly upregulated in epidemic M1T1 GAS strains (Sumby et al., 2006; Zhu et al., 2015). It is also suggested that the expression of *slo* gene is upregulated by cathelicidin antimicrobial peptide LL-37, preventing GAS to be killed by human cells (Love et al., 2012).

As a potent pore-forming toxin, SLO has been shown to lyze numerous host cells through destructing cytoplasmic membrane integrity. SLO causes lysis in eukaryotic cells by binding to membrane cholesterol in a monomeric form, followed by oligomerization and pore formation, a mechanism similarly to that mediated by staphylococcal α-toxin and lytic membrane attack complex of complement (Bhakdi et al., 1985; Duncan and Schlegel, 1975; Hugo et al., 1986; Niedermeyer, 1985; Otto, 2014; Tegla et al., 2011). Cells, such as erythrocytes, neutrophils, macrophages, platelets, and keratinocytes, are the reported targets of SLO (Duncan and Schlegel, 1975; Launay and Alouf, 1979; Sierig et al., 2003). Similar with streptolysin S, SLO contributes to β-hemolytic activity of GAS on the blood agar (Fontaine et al., 2003; Sierig et al., 2003). However, its hemolytic activity is inactivated by oxygen, while streptolysin S is oxygen-stable.

SLO contributes to GAS virulence with multiple functions. It has been demonstrated that *slo*-deficient GAS mutants decreased their virulence in various murine models of invasive diseases (Ato et al., 2008; Fontaine et al., 2003; Limbago et al., 2000; Timmer et al., 2009). More in details, studies suggested that SLO contributes to GAS virulence in the early stage of infections when GAS encounters epithelial barrier and phagocytes including neutrophils and macrophages (Ato et al., 2008; Fontaine et al., 2003; Limbago et al., 2000;

Timmer et al., 2009). In term of neutrophils, sublytic SLO sufficiently suppresses neutrophil oxidative burst, which could be reversed by free cholesterol and anti-SLO antibodies (Uchiyama et al., 2015). Furthermore, SLO impairs neutrophil degranulation, interleukin-8 secretion and antibacterial activity (Uchiyama et al., 2015). Interestingly, SLO at sublytic concentrations also inhibits neutrophil chemotaxis, possibly due to that SLO induces a rapid and massive shedding of L-selectin, the cell adhesion molecule guiding leukocytes to sites of inflammation (Walcheck et al., 1996; Walev et al., 2000; Van Epps and Andersen, 1974). However, in epidemic M1T1 clones, SLO functionally induces neutrophil lysis and cell death (Timmer et al., 2009). It could be explained by the observation that epidemic M1T1 clones typically produce a high level of SLO (Sumby et al., 2006). During neutrophil lysis, SLO apparently activates neutrophils through a p38 MAPK signaling pathway, resulting in the release of heparin-binding protein, LL-37, defensins, and elastase (Andersen and Duncan, 1980; Nilsson et al., 2006). Moreover, SLO induces the coaggregation of neutrophils and platelets in rat model, which is largely mediated by platelet P-selectin and may contributes to the vascular dysfunction and ischemic destruction observed in patients with toxic shock syndrome (Bryant et al., 2005).

Macrophages, as similar with neutrophils, response to the stimulation of recombinant SLO through activation of the NLRP3 inflammasome, leading to IL-1β secretion and pyroptosis (Keyel et al., 2013; Mitsui et al., 2002). Notably, GAS also requires SLO to activate intracellular NLRP3 inflammasome in macrophages in a caspase-1 dependent manner, independently of TLR signaling that is critical for macrophages exposed to certain bacterial pore-forming toxins (Harder et al., 2009; Mariathasan et al., 2006). This finding was constitutively mimicked by Timmer et al. who showed an accelerated, caspase-dependent SLO-induced macrophage apoptosis contributed to M1T1 GAS immune evasion and virulence (Timmer et al., 2009). Interestingly, not only caspase-1, caspases-3 and -7 are also involved in SLO-induced macrophage apoptosis, indicating that SLO rather activates multiple inflammatory programmed cell death pathways (Goldmann et al., 2009; Timmer et al., 2009). In addition to the lytic activity and modulation of

inflammation, SLO prevents internalization of GAS into lysosomes (Hakansson et al., 2005; Logsdon et al., 2011). SLO-deficient GAS was internalized directly via a clathrin-dependent pathway and rapidly transported into lysosomes, and killed through the lysosomal degradation (Hakansson et al., 2005; Logsdon et al., 2011). In contrast, SLO prevented lysosomal colocalization with intracellular GAS and acidification of GAS-containing vacuoles, and conferred GAS resist internalization and intracellular killing by pharyngeal epithelial cells (Hakansson et al., 2005; Logsdon et al., 2011). Collectively, these suggest a lysis-independent function of SLO, which is being a translocator delivering GAS toxins, in particular NADase into cell cytoplasm (Magassa et al., 2010).

2.2 NADase – an intracellular enzyme and toxin

GAS NADase or SPN is a secreted enzyme that catalyzes the hydrolysis of the nicotinamide-ribose bond of $NAD^+$ to yield nicotinamide and ADP-ribose (Ghosh et al., 2010; Grushoff et al., 1975). Because cyclic ADP-ribose is a potent second messenger for intracellular calcium homeostasis (Guse, 2004; Lee, 2001), it has been suggested that NADase could be translocated into the cytosol of host cells to deplete cellular NAD pools and disrupting cellular metabolism, leading to host cell injury and death (Bricker et al., 2005; Bricker et al., 2002; Madden et al., 2001; Michos et al., 2006).

NADase is directly toxic for a wide variety of host cells, and toxic to *E. coli* and yeast when they were employed for expression of recombinant GAS NADase (Ghosh et al., 2010; Tatsuno et al., 2007). However, NADase has no toxic effects on GAS, suggesting an alternative factor from GAS that essentially regulates the toxicity of NADase within GAS. This factor, designated as immunity factor for SPN (IFS) by Meehl et al., is an endogenous inhibitor of NADase activity (Kimoto et al., 2006; Meehl et al., 2005). IFS is co-expressed as a small protein of 161 amino acids with NADase and forms a stable complex with the later at a 1:1 molar ratio in the cytoplasm of GAS. Binding of IFS to NADase in a form of complex blocks its access to the substrate β-$NAD^+$, therefore competitively inhibiting activities of NADase and protecting GAS from exhaustion of $NAD^+$ (Kimoto et al., 2006; Meehl

et al., 2005; Smith et al., 2011). Furthermore, recombinant NADase can be normally produced in *E. coli* when co-expressed with the inhibitor IFS, and dissociated from IFS by treatment with sodium thiocyanate (Kimoto et al., 2006; Meehl et al., 2005). However, to be active and toxic to host cells, NADase need to be released from the stable complex with IFS during secretion by GAS, for which the mechanism remains to be elucidated.

NADase acts specifically as an intracellular toxin to the host. The cytotoxic effects of NADase on host requires SLO that delivers NADase to the cytoplasm of human cells, making it difficult to distinguish toxic effects of NADase from those of SLO (Karasawa et al., 1995; Magassa et al., 2010; Sharma et al., 2016). Alternatively, Sharma et al. fused active NADase to anthrax toxin, which mediates delivery of NADase into human oropharyngeal keratinocytes, independently of SLO (Sharma et al., 2016). This active NADase, but not the inactive form of NADase, induced cell death of keratinocytes. Moreover, NADase/SLO-deficient GAS had a defective intracellular survival in human keratinocytes, however, this defective survival could be recused by delivery of anthrax toxin-fused NADase into keratinocytes (Sharma et al., 2016). These indicate that NADase acts specifically as an intracellular toxin, mediating cytotoxicity and promoting intracellular survival of GAS in host cells (Bricker et al., 2002; Sharma et al., 2016). Furthermore, the toxic effects of NADase in the host are also demonstrated *in vivo* using mouse models of invasive infections, which showed a significantly attenuation of virulence in GAS lacking NADase activity (Bricker et al., 2005; Zhu et al., 2017). NADase also has an immune regulatory function through inhibiting inflammasome-dependent IL-1β release from infected macrophages, independent on SLO-mediated translocation of NADase into the host cell cytosol (Hancz et al., 2017).

### 2.3 SLO and NADase – One plus one equals more than two

As two potent toxins of GAS, SLO and NADase play relevant roles in GAS pathogenesis. More importantly, both toxins act synergistically and closely associate with each other. For example, the genes encoding SLO and NADase

are co-transcribed, while two proteins can be co-purified from the culture supernatants of GAS (Shany et al., 1973). SLO mediates the translocation of NADase into human epithelial cells, while NADase binds to SLO and enhances its activity (Michos et al., 2006; Velarde et al., 2017). Interaction between SLO and NADase seems to be a win-win event that stabilizes both toxins and increases their activities (Michos et al., 2006; Velarde et al., 2017). Furthetmore, GAS with both SLO and NADase induces an augmented cell injury of human keratinocytes (Michos et al., 2006; Velarde et al., 2017). Interestingly, SLO stimulates xenophagy in pharyngeal keratinocytes. However, SLO and NADase coordinately prevent maturation of GAS-containing autophagosomes, thereby prolonging GAS intracellular survival (O'Seaghdha and Wessels, 2013). Similarly, both toxins also mediate GAS intracellular survival and cytotoxicity for macrophages. They do not inhibit maturation of GAS-containing lysophagosomes, but rather prevent phagolysosome acidification in macrophages that may serve as reservoirs or vehicles to enable persistent GAS infection in the host (Bastiat-Sempe et al., 2014).

The synergistical effects of SLO and NADase have been elucidated not only in host cells *ex vivo* but also in various mouse models of invasive GAS infections. Bricker et al. showed that mutants deficient in SLO and NADase activity were significantly attenuated for virulence compared with the wild-type parent M3 GAS strain in mouse models of invasive soft-tissue infection and septicemia (Bricker et al., 2005). In an epidemic M1T1 GAS background, SLO and NADase had equally capacities to cause necrotizing myositis (Zhu et al., 2017). Moreover, mutants lacking SLO and NADase are impaired to resist killing by human neutrophils, and are significantly attenuated in the bacteremia and soft tissue infection models (Zhu et al., 2017). Therefore, recombinational replacement of SLO and NADase-encoded genetic region from M12 GAS contributes significantly to the emergence of contemporary epidemic M1T1 isolates.

## II. Selection of hypervirulent M1T1 GAS clone in the host

Phenotypic switching refers to a switch between two phenotypic states with

different gene expression patterns, which leads to variation in morphology, physiology, and pathogenicity of bacterial pathogens. For example, pathogen *S. aureus* switches from normal colony phenotype to a slow-growing, small colony variant phenotype that facilitates bacterial persistent in host cells and recurrent infections (Proctor et al., 2006). GAS, particularly the invasive M1T1 and M3 types, also undergoes a switch from a SpeB-positive and SpeA negative ($SpeB^+/SpeA^-$) phenotype to a $SpeB^-/SpeA^+$ phenotype when they are recovered from infected human or passaged in mouse, which is termed as *in vivo* selection (Ikebe et al., 2010; Kazmi et al., 2001; Walker et al., 2007). The *in vivo* selection, characterized as the loss of SpeB activity, has been associated with the mutations in global regulatory proteins of GAS (Carroll and Musser, 2011). GAS possesses various global regulators including two-component regulatory systems and stand-alone regulators (Kreikemeyer et al., 2003). Of these global regulators, CovRS has been well characterized (Graham et al., 2002; Sumby et al., 2006). CovRS represents a sophisticated regulatory network negatively controlling expression of multiple virulence factors including SpeB (Graham et al., 2002). However, CovRS is highly prone to spontaneous mutation in the host, which results in downregulation of SpeB production but enhancement of virulence, leading to the emergence of variants with hypervirulent/invasive capacities (Graham et al., 2002; Ikebe et al., 2010; Trevino et al., 2009).

*1. CovRS – the control of GAS virulence*

CovRS consists of a membrane-bound histidine kinase CovS and a cognate response regulator CovR. As a typical sensor histidine kinase, CovS contains a surface-exposed extracellular domain, two membrane-spanning domains, a cytosolic HAMP domain (present in Histidine kinases, Adenyl cyclases, Methyl-accepting proteins and Phosphatases), and a histidine kinase-like ATPase (HATPase) domain (Tran-Winkler et al., 2011). The extracellular domain acts to sense various environmental stimuli or stresses, *e.g.*, $Mg^{2+}$ concentration, external pH and temperature, iron starvation and LL-37 (Dalton and Scott, 2004; Froehlich et al., 2009; Gryllos et al., 2007; Gryllos et al., 2003; Gryllos et al., 2008). It is presumed that the environmental signals could influence the histidine

autophosphorylation of HATPase domain in CovS and subsequent phosphotransfer to the aspartate residue of CovR, which determines CovR phosphorylation status (Dalton and Scott, 2004; Tran-Winkler et al., 2011). CovR acts primarily as a repressor of gene expression whose phosphorylation enhances its binding to regulated promoters (Dalton and Scott, 2004; Federle and Scott, 2002; Gusa et al., 2006; Gusa and Scott, 2005; Horstmann et al., 2015; Horstmann et al., 2014; Tran-Winkler et al., 2011), mediating the cellular responses through altering distinct transcriptional profiling of GAS (Churchward, 2007; Dalton et al., 2006; Graham et al., 2002; Gryllos et al., 2007; Gryllos et al., 2003; Gusa et al., 2006; Horstmann et al., 2015; Horstmann et al., 2014; Tran-Winkler et al., 2011; Trevino et al., 2009).

In response to specific environmental stimuli, CovRS mediates distinct signal transduction and strikingly different transcriptomes. Human cationic LL-37, being a well-characterized stimulus of GAS CovRS, exhibits a broad spectrum of antimicrobial activity as well as immunomodulatory activity (Durr et al., 2006). M1T1 GAS clone is more resistant to the antimicrobial activity of human LL-37 through several mechanisms, for example, sequestering and neutralizing its activity by M1 protein, and inactivating LL-37 by the streptococcal inhibitor of complement and secreted streptococcal proteins (Durr et al., 2006; Frick et al., 2003; Johansson et al., 2008; LaRock et al., 2015; Nyberg et al., 2004). Notably, LL-37 binds directly to the extracellular domain of CovS, which dephosphorylate CovR and subsequently release the CovR-mediated repression of expression of multiple virulence factors including Sda1, SpeA2, SLO, NADase and the hyaluronic acid capsule (Gryllos et al., 2008; Love et al., 2012; Tran-Winkler et al., 2011; Velarde et al., 2014). The LL-37 signaling through CovRS leads to a marked increase of GAS virulence by promoting GAS resistance to the killing by human oropharyngeal keratinocytes, neutrophils, and macrophages (Gryllos et al., 2008; Love et al., 2012; Tran-Winkler et al., 2011). Interestingly, LL-37 signaling-upregulated GAS virulence can be adversely damped by elevated extracellular $Mg^{2+}$, although it may not be physiologically relevant (Gryllos et al., 2007; Gryllos et al., 2003; Gryllos et al., 2008; Tran-Winkler et al., 2011). The

opposite effects on expression of CovRS-regulated genes through LL-37 and $Mg^{2+}$ signaling, as an example, suggest the complexity of CovRS regulatory system, especially when invasive GAS survives in host.

*2. The spontaneous mutation of CovRS in the host*

In concert with CovRS-controlled enhanced GAS virulence elicited by host LL-37, CovRS undergoes spontaneous mutations more frequently in patients with severe GAS infections, leading to the emergence of hypervirulent/invasive variants. This naturally occurring CovRS mutation has been reported in various M types of GAS including not only M1 but also M3, M12, M28, M49 and M89 GAS clinical isolates (Ikebe et al., 2010; Uchiyama et al., 2012). However, CovRS mutations occur with a higher frequency in the invasive M1T1 isolates, which contributes to the phenotype of $SpeB^{-}$/$SpeA^{+}$ (Aziz et al., 2004b; Engleberg et al., 2001; Ikebe et al., 2010; Kansal et al., 2010). The mutations in most cases, either in CovR or CovS, in the form of nucleotide deletions, point mutations, or insertions, eventually cause dysfunction of CovRS, leading to enhanced virulence in mouse models of invasive disease (Ikebe et al., 2010; Li et al., 2014; Mayfield et al., 2014). CovRS mutation variants, for example, either generated in the laboratory or recovered after passage *in vivo*, produce elevated levels of CovRS-targeted virulent factors, especially that are involved in GAS dissemination into deep tissue and organs in the cases of necrotizing fasciitis and toxic shock syndrome (Engleberg et al., 2001; Graham et al., 2002; Horstmann et al., 2011; Kansal et al., 2010; Mayfield et al., 2014; Miyoshi-Akiyama et al., 2006; Sumby et al., 2006; Tatsuno et al., 2013; Trevino et al., 2009).

It is worth noting that CovRS mutations not only mediate the upregulation of virulence, but also modulate the cellular responses of GAS, *e.g.*, nutrient metabolism, protein biosynthesis, and environmental adaptation. More fundamentally, CovRS mutation changes the transcriptome profile of M1T1 GAS clone from a phenotype favoring epithelial colonization to the one that promotes immune evasion and dissemination of GAS (Graham et al., 2002; Hollands et al., 2010; Sumby et al., 2006). It suggests, potentially, that CovRS mutations may

shape GAS virulence in combination with other transcriptional regulators that regulate carbon source utilization and nitrogen metabolism during GAS infection (Shelburne et al., 2010). Furthermore, CovRS mutations facilitate virulence through dismissing the production of SpeB, which proteolytically degrades multiple virulence factors (Aziz et al., 2004b; Walker et al., 2007).

*3. The selection pressure for CovRS mutation variants*

What pressure or factors in the host drive the occurrence of mutations in CovRS has not been well understood. However, several studies have provided clues that help us understand the *in vivo* selection of CovRS mutations. Two types of selection pressure have been suggested to govern the mutation of CovRS in the host, one from host, another from the bacteria itself.

The selective force from the host is navigated to the innate immunity where neutrophils play a dominant role (Li et al., 2014). Neutrophils are the most abundant innate immune cells, killing invading pathogens through a comprehensive mechanism consisting of phagocytosis, respiratory burst and antimicrobial molecules-mediated killing, and NETs formation (Dohrmann et al., 2016). Compared to other bacteria, GAS is more successful to counteract the antimicrobial activities of neutrophils, which could be attributed to the observation that neutrophils provide selection pressure for the phenotypic switch of M1T1 GAS via the mutation of CovRS in the mouse infection models (Kobayashi et al., 2003; Li et al., 2014). Accordingly, such a selection was inhibited in mice whose neutrophils were depleted by specific monoclonal antibodies or when neutrophil migration was impaired during infection due to the deficiency in chemokine receptor 2 (Li et al., 2014). The recovered CovRS mutation variants from mice showed a hypervirulent phenotype as similarly as found in the clinical isolates from patients with toxic shock syndrome and other severe infections (Ikebe et al., 2010; Li et al., 2014).

GAS virulence factors also act as selective pressures that determine the CovRS mutation in the host. At least two virulence factors, M1 protein and hyaluronic acid

capsule, have been proposed being involved (Cole et al., 2010). M protein, as the major virulence factor of GAS, mediates GAS colonization of host epithelium, promotes GAS resistance to phagocytosis, inactivates LL-37 activity, as well as facilitates bacterial survival in neutrophils and NETs (Carlsson et al., 2003; Horstmann et al., 1988; Horstmann et al., 1992; LaRock et al., 2015; Lauth et al., 2009; Oehmcke et al., 2010; Staali et al., 2006). Similarly, hyaluronic acid capsule increases GAS resistance to phagocytosis and survival within NETs (Cole et al., 2010; Dale et al., 1996; Wessels et al., 1991). Both M1 protein and hyaluronic acid capsule share a common capacity that is to resist neutrophil-mediated killing (Buchanan et al., 2006; Lauth et al., 2009; Staali et al., 2003; Wessels et al., 1991). Consequently, GAS deficient in M1 protein or hyaluronic acid capsule failed to undergo phenotypic switching and was rapidly cleared by the host immune responses (Cole et al., 2010; Liu et al., 2015). Therefore, the occurrence of CovRS mutations in the host is rather a strategy for GAS to counteract host immunity, particularly the antimicrobial activities of neutrophils.

Sda1 has been also regarded as a selective force for the emergence of hypervirulent isolates in M1T1 GAS 5448 (Walker et al., 2007). Similarly as M1 protein and hyaluronic acid capsule, Sda1 confers GAS virulence through degrading NETs and increasing GAS survival in the host (Brinkmann et al., 2004; Buchanan et al., 2006; Uchiyama et al., 2012). However, horizontal transfer of the Sda1-encoding bacteriophage into the genome of M1 SF370 didn't confer GAS switch to hypervirulent *covRS* mutants in the mouse model as the phage donor strain 5448 did (Venturini et al., 2013). Moreover, we found that M1 protein, instead of Sda1, contributed to the CovRS mutation of two typical M1T1 GAS isolates in the host (Liu et al., 2015).

In a word, the selection of CovRS mutations in the host, theoretically, fits the paradigm of "Stress-Response" (Figure 1). To survive in host detrimental circumstance ("Stress"), GAS must adapt itself to evade immune attack ("Response"). It seems that GAS virulence factors including M1 protein and the capsule but not Sda1, provide primary and synergistical protection when the

M1T1 clone colonizes and invades host niches, where it survives and has opportunities to adjust the transcriptional profile through mutating in the global regulatory proteins (Cole et al., 2010; Feng et al., 2016; Liu et al., 2015; Venturini et al., 2013). Furthermore, when the detrimental circumstance or the selection pressure do not exist, GAS tends to attenuate virulence at the expense of downregulation of M protein (Zhou et al., 2013).

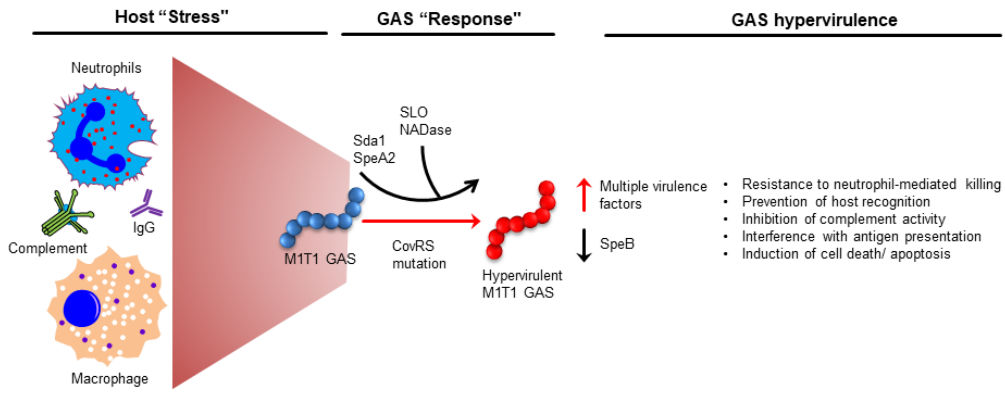


**Figure 1.** Schematic paradigm of "Stress-Response" driving the emergence of hypervirulent M1T1 GAS clone. Host innate immunity consisting of immune cells, complement and antibodies provides a "Stress" environment for GAS. To survive such a detrimental circumstance, GAS must adapt itself in response to immune stress. By acquiring genetic regions encoding Sda1, SpeA2, SLO and NADase and mutating its global regulatory proteins such as CovRS, GAS changes the transcriptional profile that promotes its hypervirulence, leading to the successful emergence of pandemic M1T1 clone.

## Concluding Remarks

The emergence of highly successful and globally disseminated M1T1 GAS clone has been identified as a threat to human health. This review highlights the importance of genetic recombination events, through which M1T1 GAS obtains virulence factors including Sda1, SpeA2, SLO and NADase. Further, this review highlights the significance of selection pressures from either neutrophils or bacteria, that modulate the upregulation of GAS virulence via the mutation of CovRS. Finally, genetic recombination alongside with the selective force

cooperatively drive the emergence of successful pandemic M1T1 isolates. By understanding the evolutionary pressures that select and shape the hypervirulent M1T1 clone, we could potentially develop new therapeutic strategies to tackle challenges when dealing with the globally disseminated M1T1 GAS clone.

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