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Toxins and Superantigens of Group A Streptococci

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ABSTRACT *Streptococcus pyogenes* (i.e., the group A *Streptococcus*) is a human-restricted and versatile bacterial pathogen that produces an impressive arsenal of both surface-expressed and secreted virulence factors. Although surface-expressed virulence factors are clearly vital for colonization, establishing infection, and the development of disease, the secreted virulence factors are likely the major mediators of tissue damage and toxicity seen during active infection. The collective exotoxin arsenal of *S. pyogenes* is rivaled by few bacterial pathogens and includes extracellular enzymes, membrane active proteins, and a variety of toxins that specifically target both the innate and adaptive arms of the immune system, including the superantigens; however, despite their role in *S. pyogenes* disease, each of these virulence factors has likely evolved with humans in the context of asymptomatic colonization and transmission. In this article, we focus on the biology of the true secreted exotoxins of the group A *Streptococcus*, as well as their roles in the pathogenesis of human disease.

Streptococcus pyogenes (i.e., the group A *Streptococcus*) is a human-restricted and versatile bacterial pathogen that produces an impressive arsenal of both surface-expressed and secreted virulence factors. *S. pyogenes* exists primarily as an asymptomatic colonizer of the skin and mucous membranes of the nasopharynx, and despite being universally sensitive to β-lactam antibiotics *in vitro*, this bacterium continues to generate significant morbidity and mortality on a global scale. Human diseases induced by *S. pyogenes* usually occur as relatively uncomplicated manifestations such as pharyngitis and skin infections, but it may also cause more problematic diseases including erysipelas and scarlet fever. In addition, *S. pyogenes* can cause devastating invasive diseases including puerperal sepsis, bacteremia, necrotizing fasciitis, and streptococcal toxic shock syndrome (TSS). This bacterium is further recognized as a very important cause of postinfection sequelae including acute rheumatic fever and rheumatic heart disease, acute glomerulonephritis, and potentially, pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections. Although surface-expressed virulence factors are clearly vital for colonization, establishment of infection, and the development of disease, the secreted virulence factors are likely the major mediators of tissue damage and toxicity seen during active infection. The collective exotoxin arsenal of *S. pyogenes* is rivaled by few bacterial pathogens and includes extracellular

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enzymes, membrane active proteins, and a variety of toxins that specifically target both the innate and adaptive arms of the immune system, including the superantigens; however, despite their role in *S. pyogenes* disease, each of these virulence factors has likely evolved with humans in the context of asymptomatic colonization and transmission. In this article, we will focus on the biology of the true secreted exotoxins of the group A *Streptococcus*, as well as their roles in the pathogenesis of human disease.

THE CYTOLYTIC TOXINS OF *S. PYOGENES*

β-hemolytic activity on blood agar is a hallmark feature of the group A *Streptococcus*, and this phenotype has been used clinically for over a century. The streptolysin S (SLS) exotoxin is a small, oxygen stable hemolysin that is responsible for the characteristic β-hemolysis of *S. pyogenes* (1). SLS is a cytolytic toxin that has features of the ribosomally synthesized and posttranslationally modified lantibiotic bacteriocins from other Gram-positive bacteria; however, lantibiotic bacteriocins mostly function as antibacterials without cytotoxic activity for eukaryotic cells (2). Production of SLS is encoded by the SLS-associated gene (*sag*) cluster, which contains genes encoding the structural SLS protein (*sagA*) as well as enzymes responsible for the posttranslational modification and transport of the toxin and, potentially, an immunity function (*sagB* through *sagI*) (3). Furthermore, the *sag* locus is conserved among group A *Streptococcus* strains regardless of M protein serotype (3). SLS has been shown to target and disrupt the function of multiple host cells, including erythrocytes, macrophages, neutrophils, and keratinocytes (2, 4–6). The exact mechanism of action of SLS is not completely clear, although it is thought to act by forming a pore in host cell membranes (7). However, a recent study has demonstrated that SLS lyses erythrocytes by disrupting the function of anion transporters to cause an influx of chloride anions (Cl^-). The influx of Cl^- is followed by an influx of water, resulting in colloidal-osmotic rupture of erythrocytes (8). Furthermore, SLS has been shown to work in conjunction with the host protease calpain to facilitate the destruction of intracellular junction proteins, promoting paracellular invasion of the bacteria across the epithelial barrier (9). To examine the role of SLS in invasive models of disease, infection of an *S. pyogenes* strain deleted for SLS function was tested in a murine model of necrotizing soft tissue infection, and SLS was determined to be essential for pathogenesis (3). A similar study using transposon mutagenesis created a mutant deficient in SLS production that remained normal with respect to other exoprotein expression and was also shown to be markedly less virulent in a mouse model of subcutaneous infection than in the isogenic wild-type strain (10). Therefore, SLS likely contributes to *S. pyogenes* pathogenesis through a combination of inhibiting phagocyte function and damaging epithelial barriers.

Streptolysin O (SLO) is a well-characterized exotoxin that functions as a thiol-activated cytolysin and is secreted from nearly all group A *Streptococcus* isolates (11). The *slo* gene encodes SLO monomers that oligomerize on eukaryotic membranes in a cholesterol-dependent manner, resulting in the formation of a large pore. This produces significant damage and ultimately results in host cell apoptosis (12). The SLO toxin is active in a reduced state and is rapidly inactivated in the presence of oxygen; thus, activity is not thought to be visible on routine blood agar plates (13). Previous studies have shown that the host targets of SLO include erythrocytes, macrophages, neutrophils, and keratinocytes (14–17). SLO has also been shown to inhibit neutrophil degranulation at sublytic concentrations (16). In addition to SLO, the *slo* gene resides in an operon that additionally encodes *S. pyogenes* NADase (SPN). The NADase functions by hydrolyzing cellular NAD^+ and by depleting cellular ATP to decrease the host energy sources (18). Multiple studies have concluded that the entry of SPN into host cells is dependent on SLO pore formation (19, 20), and SPN and SLO in conjunction increase epithelial cell apoptosis, compared to SLO alone (21). Using immortalized human keratinocytes, strains lacking SLO were attenuated for intracellular survival compared to the wild-type strain (20). Furthermore, in a murine model of necrotizing soft tissue infection, *S. pyogenes* strains lacking SLO were deficient in causing myositis, bacteremia, and soft tissue infection (22). Another study demonstrated lower infectivity of SPN mutant strains in models of necrotizing fasciitis, although the deletion of SLO led to an inability to cause disease (22). Therefore, SLO likely promotes the pathogenesis of *S. pyogenes* in invasive skin and soft tissue infection through the destruction of phagocytes, as well as the cytolysin-mediated translocation of SPN into target cells.

Independent studies investigating the role of both SLO and SLS in murine models of necrotizing fasciitis and systemic infection each demonstrated that both cytolytic toxins significantly contribute to the development of murine necrotizing fasciitis (23, 24). Although SLO and SLS have been extensively studied and shown to be important contributors to the pathogenesis of invasive *S. pyogenes* infections, their roles during coloniza-

tion and acute nasopharyngeal infection have yet to be defined.

STREPTOCOCCAL SUPERANTIGENS

S. pyogenes is recognized as one of the few bacterial pathogens that produces superantigen exotoxins. Streptococcal superantigens are ribosomally synthesized, relatively low molecular mass (~22 to 28 kDa) proteins that contain classical signal peptides that are cleaved after secretion to release the mature toxin. Superantigens function by activating T cells and are among the most potent known activators of T cells. The term "superantigen" was first used to describe the massive primary T cell response to these bacterial toxins (25). However, the *S. pyogenes* superantigens are also historically known as the erythrogenic toxins or scarlet fever toxins, due to their role in causing a red rash in the context of scarlet fever (26–28). In fact, the characteristic rash seen in scarlet fever is likely due to an amplified hypersensitivity reaction resulting from superantigen activity (28). Since the appearance of a rash was the defining feature of the "erythrogenic" toxins, Watson (27) first proposed the name "streptococcal pyrogenic exotoxin" (Spe) owing to the fever-producing ability, because he initially believed that the Spes belonged to a separate family of toxins. We now know that these represent the same group that belongs to a larger group of structurally conserved exotoxins that are also produced by *Staphylococcus aureus* (29–32) and some Lancefield group C and G β-hemolytic streptococci (33–37). There are now at least 14 characterized and genetically distinct *S. pyogenes* superantigens, and many of them are encoded within lysogenic bacteriophage, or putative bacteriophage, elements. Therefore, different strains often encode different repertoires of typically between 3 and 6 distinct superantigen genes (38–42). Although nomenclatural inconsistencies exist in the literature, a recent comprehensive review of the streptococcal superantigens has clarified many of these issues (43). Using this updated nomenclature, the currently known repertoire includes streptococcal pyrogenic exotoxin (Spe) types A, C, G, H, I, J, K, L, M, N, O, and P, as well as the streptococcal superantigen and streptococcal mitogenic exotoxin Z (SmeZ) (Fig. 1A). The SpeN, SpeO, and SpeP superantigens were first identified in *Staphylococcus equi* subsp. *zooepidemicus* (originally defined as SzeN, SzeF, and SzeP, respectively) (36), but to our knowledge, these particular superantigens have not been identified in *S. pyogenes*. Additionally, *S. equi* subsp. *equi* can encode SeeH, SeeI, SeeL, and SeeM, which are highly orthologous to SpeH, SpeI, SpeL, and SpeM, respectively (37). Although most of the streptococcal superantigens are encoded on bacteriophage elements, SpeG and SmeZ are exceptions in that they appear to be encoded within the core chromosome (35, 38–42). SpeJ is also not found in association with bacteriophage genes (40), yet it is not present in the many sequenced strains. SmeZ, in particular, is known to have many allelic variants, and most of the sequence changes are located on the surface of the toxin and rarely found within the predicted receptor binding domains. This indicates that SmeZ is likely under significant immunological pressure to alter antigenic characteristics as a possible immune evasion strategy (35). Despite the fairly weak primary amino acid sequence homology (Fig. 1B), crystal structures determined for these toxins share the "generic" superantigen fold (Fig. 1C) (44). Each structure includes an N-terminal α-helix which leads into a β-barrel domain, also known as the oligosaccharide/oligonucleotide binding fold (45). A central α-helix then joins this domain to a β-sheet structure known as the β-grasp domain. Despite the clear similarities to the overall fold, there are also important differences in how these toxins engage their host receptors.

Superantigens function by causing excessive T cell activation through simultaneous engagement of both the variable region of the T cell antigen receptor (TCR) β-chain (referred to as "Vβ") on T cells and to different regions of major histocompatibility complex (MHC) class II molecules on antigen-presenting cells (44, 46). Thus, superantigens are remarkable in that they have evolved to target two critical and extremely diverse receptors of adaptive immunity. In superantigen-mediated T cell activation, the toxin binds directly to MHC class II molecules, and this occurs without processing by the antigen-presenting cell. Superantigens do not undergo major modifications after release from the cell or major structural alterations upon binding to their ligands. The interaction with the TCR is dependent on binding to different Vβ regions, and this occurs, generally, away from the regions of the TCR that are critical for peptide-specific recognition. Due to the unconventional contacts created by these interactions, superantigen-mediated T cell activation is also not MHC restricted, and furthermore, both $CD4^+$ and $CD8^+$ T cells are stimulated in a Vβ-specific manner. Thus, superantigens can "force" an excessive primary response that is not seen with conventional peptide antigens. In the most severe case, this excessive activation of T cells results in the massive release of proinflammatory cytokines from both T cells and antigen-presenting cells (47) that can result in a serious and potentially lethal disease defined as TSS (32).

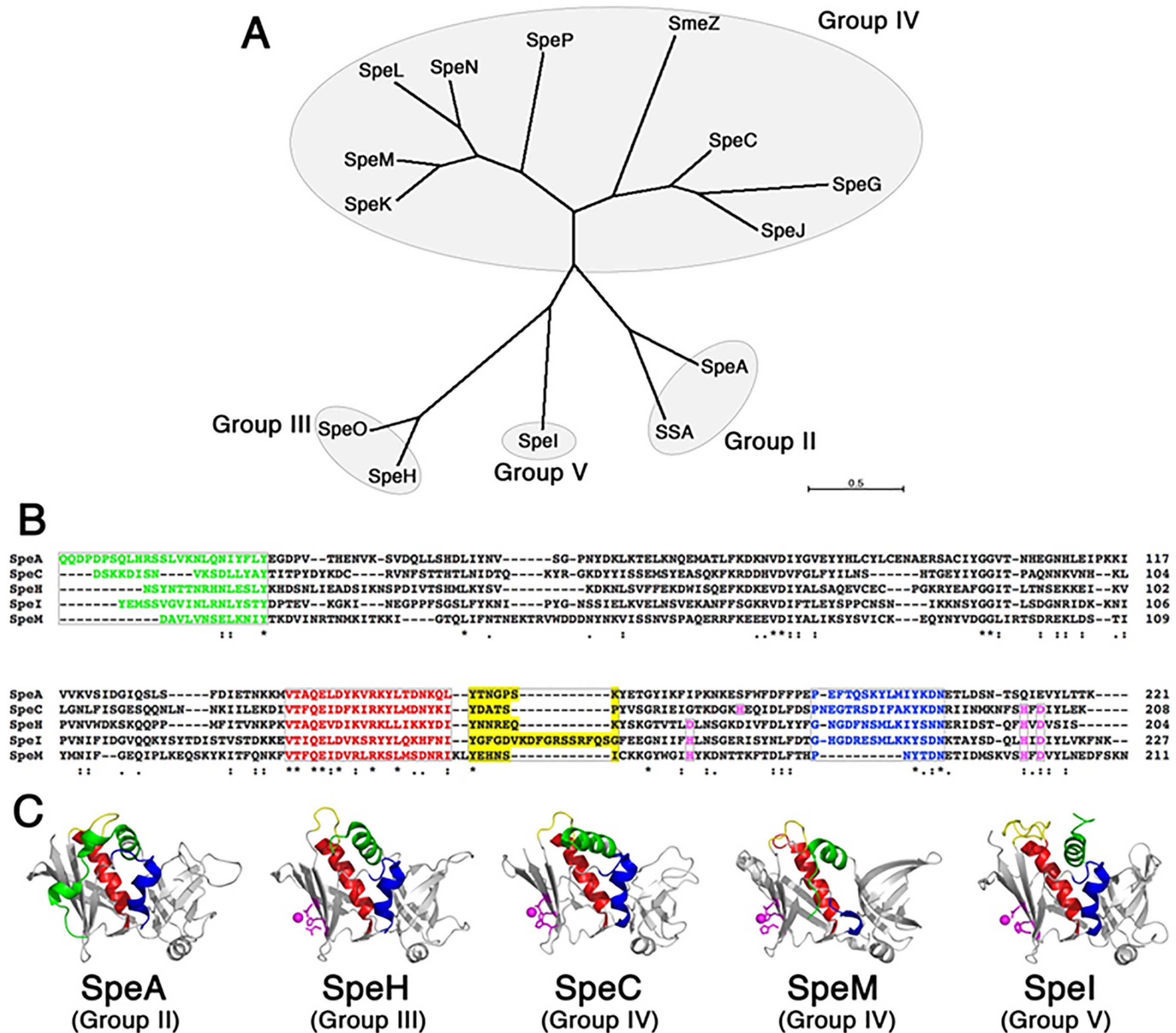


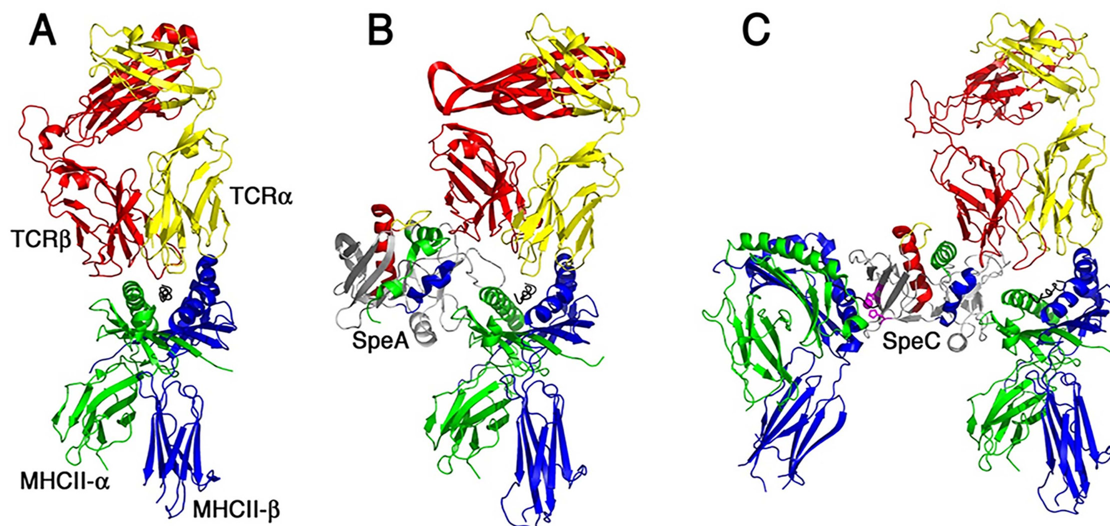
FIGURE 1 Phylogenetic relationships and structural conservation of the streptococcal superantigens. **(A)** Unrooted neighbor-joining tree showing phylogenetic relationships of known streptococcal superantigens. The unrooted tree was based on the alignment of amino acid sequences using CLUSTAL W (166) and constructed using MEGA7 (167). The groups indicate a prior classification scheme for the superantigen family (32). **(B)** Amino acid alignment of five representative streptococcal superantigens. The colors designate distinct domains in the superantigen structure, including the N-terminal α-helix (green), the central α-helix (red), the α3-β8 loop that is unique to the group V superantigens (168), and a C-terminal α-helix that is lacking in a subgroup of group IV. Residues involved in the coordination of a zinc atom important for binding to the MHC class II β-chain are colored magenta. **(C)** Crystal structures of representative streptococcal superantigens are colored as in panel B.

Cocrystallization structures have been determined for SpeA and SpeC in complex with the mouse Vβ8.1 and human Vβ2.1 chains, respectively (48). Earlier cocrystal structures exist for the staphylococcal superantigens staphylococcal enterotoxin B (SEB) and staphylococcal enterotoxin C (SEC) in complex with the mouse Vβ8.1 chain (49, 50). Although highly homologous superantigens, such as SEB, SEC, and SpeA, may have similar architectures for their Vβ chain interactions, the β-chain-SpeC complex involved a significantly larger contact surface, a different orientation, and contacts with the complementarity determining region (CDR) 3 loop, whereas SEB, SEC, and SpeA had no intermolecular contacts with this loop (48). Nevertheless, it appears that the critical component for functional recognition of the TCR is the CDR 2 loop of the β-chain (51).

The interaction of superantigens with MHC class II can also show considerable diversity, because superantigens have two independent binding domains. One binding face occurs through the N-terminal oligosaccharide/oligonucleotide binding fold domain and is of relatively low affinity (~10^{-5} M). Although somewhat controversial (52–55), it appears likely that most, if not all, streptococcal superantigens contain this low-affinity MHC binding domain. A second, zinc-dependent MHC binding face occurs on the β-grasp domain and is of relatively high affinity (~10^{-7}). The zinc ion, shown in magenta in Fig. 1C, is coordinated through three conserved residues, whereas the fourth ligand is from a conserved residue on the MHC β-chain.

Figure 2 presents the complexes for a conventional TCR-MHC class II interaction, as well as how SpeA (which lacks a high-affinity MHC class II binding domain) likely engages TCR Vβ and MHC class II and SpeC, which crosslinks MHC class II (56). The SpeA model is analogous to the standard "wedge" model of superantigen-mediated T cell activation (49), whereas the SpeC model is analogous to the proposed model for

FIGURE 2 Models of T cell activation complexes for streptococcal superantigens. Ribbon diagrams demonstrating typical antigen-mediated T cell activation **(A)** and modeled T cell activation complexes for SpeA **(B)** and SpeC **(C)**. The cocrystal structures of SpeA and SpeC in complex with their respective TCR β-chains (48) and of SpeC in complex with the MHC class II through the zinc-dependent high-affinity binding domain have been determined (169). SpeC also activates T cells in a mode similar to the staphylococcal enterotoxin A model (58) where SpeC engages MHC class II α-chain through a generic low-affinity binding domain (170) and engages the MHC class II β-chain through a zinc-dependent, high-affinity binding domain (169). The binding architecture for the generic low-affinity MHC class II binding to SpeA and SpeC is modeled using the staphylococcal enterotoxin B-MHC class II cocrystal structure (171). Note the presence of the zinc ion (magenta) coordinated in the high-affinity binding site for SpeC and that SpeA lacks this zinc site. The TCR α-chain (shown in gray) for both the SpeA and SpeC diagrams is modeled for clarity by superimposition of the α/β TCR shown on the left of the respective TCR β-chains for both superantigens. The figure was generated using Pymol.



staphylococcal enterotoxin A (57, 58). In these models, the mechanism by which superantigens subvert the normal T cell activation process is apparent, whereby the antigenic peptide is displaced from the TC, and the specificity of the reaction is governed by superantigen recognition of the Vβ CDR2 loop (51).

Since early in the past century, the incidence of serious streptococcal disease greatly decreased, likely owing to the widespread use of antibiotics (59). However, it was not until the late 1980s that descriptions of well-defined cases led to the recognition of a severely toxic streptococcal superantigen-mediated disease called streptococcal TSS (60, 61). Indeed, since the mid-1980s there has been a sustained resurgence in the frequency and severity of streptococcal TSS and other invasive diseases caused by group A streptococci (62–64). This led to the hypotheses that there was an emergence of an excessively virulent strain or strains or, alternatively, that there had been major changes in host susceptibility to these infections. Indeed, many of the contemporary invasive infections have been attributed to the M1T1 clone (62), which is now recognized to have acquired an $SpeA_2$- and DNAse-encoding bacteriophage, as well as a horizontally acquired region encoding the SLO and SPN, likely from an M12 serotype (65). These acquisitions increase the virulence of this clone (66). Over the past decade, there has also been a dramatic resurgence in scarlet fever in mainland China and Hong Kong, South Korea, the United Kingdom, and Germany (67–70). Although it is not yet entirely clear, these large-scale outbreaks are likely linked to the acquisition of superantigen-producing bacteriophage elements in *S. pyogenes* (71–73).

Multiple lines of experimental evidence indicate that the streptococcal superantigens are significant virulence factors for severe streptococcal disease. The prototypical streptococcal superantigens are SpeA and SpeC, and these two toxins have been recognized as the likely causative agents in most cases of streptococcal TSS (61, 62, 74). Indeed, SpeA and SpeC are produced by many *S. pyogenes* strains isolated from patients with streptococcal TSS (29, 74–78). Due to the association of SpeA and SpeC with streptococcal TSS, two toxoid vaccines have been generated for these toxins that, when used in the rabbit model of TSS, provided complete protection to lethal quantities of the respective superantigen (53, 79). There are at least six naturally occurring alleles of *speA* (80, 81), and serotype M1 and M3 strains expressing the $speA_2$ and $speA_3$ alleles caused the majority of streptococcal TSS in the 1980s (81). Of the streptococcal superantigens that have been tested, the toxins share the properties of inducing fever (pyrogenicity) and lethal shock in the rabbit model. For example, recombinant SpeA, SpeC, and SpeJ can induce symptoms of streptococcal TSS without necrotizing fasciitis/myositis in this model (53, 79, 82), and SpeA administered to rabbits as a vaccine also conferred significant protection from TSS with necrotizing fasciitis/myositis after challenge with viable M1 and M3 streptococci (64). Circulating superantigens have also been found in patients with streptococcal TSS (83), and the lack of humoral immunity to the superantigens is a risk factor for the development of streptococcal TSS (84, 85).

Although significant immunological research with bacterial superantigens has been conducted in mouse models, conventional mice are no longer considered appropriate models to evaluate the biological effects of these toxins. Lethal effects are not seen at very high relative doses without the coadministration of a liver-damaging agent, whereas the rabbit model is susceptible to fairly low doses when administered continuously (86). It has further been demonstrated that HLA-DQ transgenic mice were more susceptible to SpeA-induced lethality whether administered as a pure toxin or during infection with $SpeA^+$ *S. pyogenes*, whereas nontransgenic mice did not show an obvious effect (87, 88). Consistent with this, it has been established that in humans, MHC class II allelic variation contributes to the differences in severity seen for invasive streptococcal infections (89). This is understood to be due to differential cytokine production triggered by streptococcal superantigens, and MHC class II α-chain polymorphisms affect superantigen responses (90, 91). Recently, using infection models with transgenic mice expressing human MHC class II alleles, we demonstrated that the SpeA superantigen is a critical bacterial factor that is necessary to induce an acute nasopharyngeal infection (92). Using this model, SpeA was shown to activate specific Vβ-subsets *in vivo* (93), and strikingly, functional Vβ-specific T cell subsets were required for the infection phenotype (94). Furthermore, humoral immunity to the SpeA superantigen dramatically inhibited infection by *S. pyogenes* (92, 94). We believe that these findings support the further development of toxoid superantigens as vaccines to target the transmission and colonization by *S. pyogenes*.

STREPTOCOCCAL PYROGENIC EXOTOXIN B

SpeB is highly conserved in virtually all *S. pyogenes* isolates (95, 96), although despite its original name as a streptococcal pyrogenic exotoxin, SpeB is known to function as a broad-spectrum protease and is secreted as a 40-kDa zymogen. The pro-domain is auto-catalytically

cleaved to generate an active 28-kDa proteinase (97, 98). Additionally, an SpeB inhibitor located downstream of *speB* is cotranscribed with the protease. The inhibitor, termed Spi, has sequence and predicted structural homology with the pro-domain of SpeB (99). The inhibitor is resistant to cleavage by active SpeB, making it an effective inhibitor of protease activity within the bacterial cytoplasm (99). Although SpeB is highly conserved across most *S. pyogenes* strains, its expression may vary depending on the type of infection (100). For example, when analyzing isolates from patients with pharyngitis, impetigo, invasive disease, or acute rheumatic fever, 40% of isolates from patients with acute rheumatic fever produced SpeB, compared to only 5.5% of isolates from impetigo patients (100). Therefore, the variability in SpeB expression suggests that SpeB is more important for certain forms of disease progression.

SpeB is considered an important virulence factor most likely due to the immunomodulating effects of its protease activity (101, 102). SpeB has broad proteolytic activity against a number of human proteins, including interleukin-1β (102), immunoglobulins (101), fibrinogen (103), fibronectin (104), kininogens (105), and a metalloprotease (106). Although SpeB has been shown to cleave certain immunoglobulins, a study investigating the cleavage of IgG under physiologic conditions found that IgG is only cleaved in a reduced form, whereas physiologic IgG is active in an oxidized form (107). Therefore, SpeB likely does not contribute to *S. pyogenes* pathogenesis through the cleavage of IgG. The innate host immune system is also weakened through proteolytic destruction of complement factors such as C3 and the membrane attack complex (108, 109). SpeB also has the capacity to cleave many human chemokines (110), further disrupting the immune response. In addition to the many immune modulating factors degraded by SpeB, the cysteine protease also degrades tight-junction proteins such as E-cadherin and desmogleins, facilitating the paracellular invasion of the bacteria across the epithelial barrier (111, 112). Therefore, the broad spectrum of SpeB targets likely ameliorates the virulence of *S. pyogenes* by perturbing the host immune system and compromising the epithelial barrier.

Given the broad spectrum of SpeB, *S. pyogenes* proteins can also be proteolyzed (113). SpeB can directly degrade multiple superantigens, such as SmeZ, which affects the ability of the superantigen to induce proliferation of human lymphocytes (114, 115). In addition, the cleavage of protein F1, an *S. pyogenes* cell wall-attached fibronectin-binding protein, decreased the fibronectin-dependent internalization of *S. pyogenes* into human cells, suggesting that SpeB also plays a role in the internalization process (116). Likewise, SpeB-deficient mutants were shown to have enhanced *in vitro* internalization into human epithelial and endothelial cells (117). Therefore, an inactive SpeB may be favored later during infection to promote the dissemination of the bacteria (116). Furthermore, earlier work identified a phase shift in an invasive M1T1 strain *in vivo* favoring a nonfunctional cysteine protease to conserve the function of the multiple virulence factors normally degraded by SpeB (118, 119). This phase shift is linked to inactivating mutations within the two-component CovRS sensor kinase, which results in a hypervirulent phenotype in the serotype M1 background (120). An inverse relationship has also been identified between SpeB expression and the severity of invasive group A streptococci infections, further suggesting that *S. pyogenes* may need to differentially regulate SpeB expression, depending on the site of infection and events occurring during infection (121).

In vivo studies supporting the role of SpeB as a virulence factor have not always been congruent. For example, an *S. pyogenes* strain with decreased SpeB expression exhibited a decreased capacity to cause murine necrotizing fasciitis, and complementation of SpeB restored the wild-type phenotype (122). Alternatively, isogenic gene replacement strains and a mouse model of invasive soft tissue infection showed that *speB* mutants had no apparent effect on the ability of group A streptococci to cause local tissue injury and invasive infection (123). Overall, the diversity of SpeB targets may contribute to the protease being both a virulence factor and a form of virulence regulation through the degradation of bacterial proteins.

DNases OF *S. PYOGENES*

The DNases of *S. pyogenes* constitute an additional mechanism that contributes to the pathogenicity of the bacterium. Through the analysis of all known *S. pyogenes* genomes, eight DNases have been identified (124): *spnA*, *spdB*, *sda1*, *sda2*, *spd1*, *spd3*, *spd4*, and *sdn*. Of the known DNases, *spnA* and *spdB* are chromosomally encoded and are found across all *S. pyogenes* isolates (125, 126). The six other DNases are prophage associated, and therefore they are only found in certain *S. pyogenes* strains (124). Furthermore, the DNase *spnA* is the only known cell wall-anchored DNase of *S. pyogenes*. (124, 126).

DNases contribute to the pathogenesis of *S. pyogenes* by facilitating innate immune evasion of the pathogen. During infection, neutrophils release antibacterial granule proteins and chromatin to create neutrophil extracellular traps (NETs) (127). NETs bind and trap bacteria and

degrade bacterial virulence factors, ultimately resulting in bacterial death (127). The secreted DNase Sda1 and the cell wall-anchored DNase SpnA are both able to degrade the chromatin backbone of NETs, allowing the bacteria to avoid the degradation of their virulence factors and promote bacterial survival (128–130). *S. pyogenes* DNases further contribute to pathogenesis through the degradation of bacterial DNA (131). By degrading CpG-rich bacterial DNA, the TLR9-mediated immune response is weakened due to the degradation of the TLR9 activating signal (131). In a model of mouse necrotizing fasciitis, Sda1 was shown to suppress TLR9-dependent tumor necrosis factor-α and tumor necrosis factor-α induction, promoting evasion of the innate immune system (131). Additionally, a murine model of skin and soft tissue and a cynomolgus macaque model of pharyngitis infection showed that isogenic mutants of *S. pyogenes* lacking DNase production were less virulent than the wild-type strain, highlighting their importance in pathogenesis (129). The ability of DNases to degrade NETs and bacterial DNA, along with the decreased virulence of DNase-deficient mutants *in vivo*, emphasize the importance of *S. pyogenes* DNases as extracellular virulence factors.

STREPTOCOCCAL INHIBITOR OF COMPLEMENT

The streptococcal inhibitor of complement (SIC) is a 31-kDa protein secreted by certain *S. pyogenes* strains, most notably M1 serotypes (132). SIC is highly variable, with large numbers of variants arising *in vivo* during epidemic spread of the organism (133, 134). Such high variation suggests that the phenomenon offers selective advantage to the microbe. Most of the variants are single amino acid changes, insertions, or deletions. Other M types may make related proteins, some of which have minimal effect on the complement system (135). SIC was originally identified as a protein that inhibits the membrane attack complex of the complement system, with its mechanism being the inhibition of C567 insertion into the membrane (136). Furthermore, SIC is rapidly internalized by neutrophils and binds specifically to ezrin and moesin, proteins that link the actin cytoskeleton to the host cell surface, interfering with polymorphonuclear opsonophagocytosis and intracellular killing (136). SIC also directly inhibits other components of the innate immune system, including lysozyme, secretory leukocyte proteinase inhibitor, human defensins, and cathelicidin LL-37, all of which can be toxic to *S. pyogenes* (137–140). Additionally, SIC inhibits the release of bradykinin, a potent vasodilator, which could function to decrease inflammation at the site of infection, allowing the bacteria to persist (141). More recently, SIC also inhibited the bactericidal activity of histones, which are found in NETs (142).

In vivo studies suggest that SIC increases streptococcal virulence. Mice that were inoculated intranasally with M1 SIC-negative streptococci had a lower incidence of throat colonization than mice inoculated with the wild-type M1 strain (143). SIC-negative strains also exhibited a decrease in survival in macrophages (137). Therefore, SIC likely promotes bacterial survival and dissemination by allowing the organisms to avoid innate immune defenses in the extracellular environment.

IgG-TARGETING ENZYMES OF *S. PYOGENES*

S. pyogenes has evolved multiple mechanisms to target IgG antibodies, a major effector molecule of the humoral immune system. The immunoglobulin G-degrading enzyme (IdeS, also known as Mac) is a protease that removes the Fc region of IgG antibodies and can thus inhibit opsonophagocytosis of *S. pyogenes* (144, 145). This virulence factor is specific for IgG and does not target IgM, IgE, or IgD antibodies (144). However, *in vivo* studies using a mouse invasive infection model failed to demonstrate a contribution of IdeS to virulence (146). Endoglycosidase S (EndoS) is an ~108-kDa secreted enzyme that hydrolyzes the β-1,4 linkage between the first two *N*-acetylglucosamine residues on the glycan linked to Asn297 within the C_H2 domain of the IgG Fc region (147). Deglycosylation of IgG through this mechanism impairs antibody effector functions, including binding to the Fc-gamma receptor and complement activation (148). Nevertheless, similar to IdeS, deletion of the gene encoding EndoS in the M1 5448 background had no obvious phenotype in an invasive intraperitoneal mouse infection model (149). In both cases, the lack of an *in vivo* phenotype may reflect a more subtle role for these enzymes that is not apparent through acute, invasive infections. In addition to EndoS and IdeS, which show high levels of specificity for IgG, the broad-spectrum SpeB cysteine protease is also capable of cleaving IgG in the hinge region, similar to protease papain (147); however, the *in vivo* relevance of this has been questioned (107).

STREPTOKINASE

Streptokinase is a single-chain 414-amino acid protein that is able to activate the host protein plasminogen

(150). Streptokinase forms a complex with the inactive zymogen plasminogen, or a trimolecular complex with plasminogen and fibrinogen, to generate the active serine protease plasmin (151, 152). These activator complexes bind to the bacterial surface, allowing the bacteria to acquire additional protease activity. Plasmin is responsible for degrading fibrin clots, connective tissue, extracellular matrix components, and adhesion proteins (153). Therefore, the improper activation of plasmin could result in widespread tissue damage and dissemination of the bacteria (154, 155). Transgenic mice expressing human plasminogen had an increased mortality rate compared to mice without the transgene. This susceptibility was directly related to streptokinase expression, highlighting the importance of streptokinase in *S. pyogenes* virulence (154). Furthermore, increased levels of bacteria-bound plasmin correlated with a decrease in C3b deposition and a decrease in C3b-mediated neutrophil killing (156). Additionally, bacteria-associated plasmin has been shown to prevent histone-mediated killing by NETs (157). In addition to activating plasminogen, streptokinase has also been shown to activate the contact system, resulting in the release of bradykinin (158). The release of bradykinin triggers vascular leakage, which could further promote the dissemination of the bacteria (159, 160). The release of streptokinase by *S. pyogenes* ultimately promotes the dissemination of the bacteria by facilitating the conversion of plasminogen to plasmin.

FUTURE PERSPECTIVES

The increased incidence of severe invasive group A streptococcal diseases, and the recent resurgence of scarlet fever, still remain incompletely explained; however, the streptococcal superantigens are clearly implicated in both illnesses and thus are key virulence factors driving the evolution of *S. pyogenes* pathogenesis. Indeed, the majority of genetic diversity in different strains and serotypes of *S. pyogenes* occurs due to the presence or absence of large mobile genetic elements, including lysogenic bacteriophage and integrative conjugative elements (161, 162). In particular, many of the superantigens, as well as DNases, and some other select toxins are encoded within bacteriophage elements, and this provides *S. pyogenes* with an extra level of adaptability. The apparent excessive redundancy of these toxins has yet to be explained, although we believe this allows *S. pyogenes* to avoid neutralizing antisuperantigen antibodies and may also provide additional means to efficiently target polymorphic MHC class II molecules from different populations.

Although most research on streptococcal exotoxins has focused on their role in severe streptococcal diseases, the established niche for *S. pyogenes* is a state of colonization in the throat or on the skin of humans. Also, multiple *S. pyogenes* virulence factors do not operate efficiently in mouse models (92, 154, 163–165). To understand the basic biology of this organism, which is not related to severe and invasive disease, better models will be necessary to evaluate specific virulence factors, therapies, and vaccines. Thus, most, if not all, of these remarkable exotoxins that can alter normal immune system function, damage tissue, and promote disease have each likely evolved in the context of streptococcal persistence and transmission. We hope a clearer understanding will lead to further rationales to design vaccines capable of targeting the colonization state of *S. pyogenes*.

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