Staphylococcus aureus: The Toxic Presence of a Pathogen Extraordinaire

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Abstract: Staphylococcus aureus is a facultative, Gram-positive coccus well known for its disease-causing capabilities. In particular, methicillin and vancomycin resistant strains of S. aureus (MRSA and VRSA, respectively) isolated globally represent daunting medical challenges for the 21st Century. This bacterium causes numerous illnesses in humans such as food poisoning, skin infections, osteomyelitis, endocarditis, pneumonia, enterocolitis, toxic shock, and autoimmune disorders. A few of the many virulence factors attributed to S. aureus include antibiotic resistance, capsule, coagulase, lipase, hyaluronidase, protein A, fibronectin-binding protein, and multiple toxins with diverse activities. One family of protein toxins is the staphylococcal enterotoxins (SEs) and related toxic shock syndrome toxin-1 (TSST-1) that act as superantigens. There are more than twenty different SEs described to date with varying amino acid sequences, common conformational, and similar biological effects. By definition, very low (picomolar) concentrations of these superantigenic toxins activate specific T-cell subsets after binding to major histocompatibility complex class II. Activated T-cells vigorously proliferate and release proinflammatory cytokines plus chemokines that can elicit fever, hypotension, and other ailments which include a potentially lethal shock. In vitro and in vivo models are available for studying the SEs and TSST-1, thus providing important tools for understanding modes of action and subsequently countering these toxins via experimental vaccines or therapeutics. This review succinctly presents the pathogenic ways of S. aureus, with a toxic twist. There will be a particular focus upon the biological and biochemical properties of, plus current neutralization strategies targeting, staphylococcal superantigens like the SEs and TSST-1.

Keywords: Animal model, cytokine, immunotherapeutic, receptor, Staphylococcus aureus, superantigen, toxic shock, vaccine.

1. INTRODUCTION TO A MICROBIAL TOXIN FACTORY

Staphylococcus aureus is one of the more formidable, disease-causing bacteria affecting humans and livestock [1-3]. The latum-derived genus species name means “golden cluster seed” for this facultative anaerobe that grows in grape-like bunches yielding yellow colonies on agar. S. aureus was first discovered by Alexander Ogston in 1880 following microscopy of a patient’s pus-filled abscess. The bacterium readily colonizes skin as well as various mucosal surfaces, and in most cases S. aureus provokes few problems with its host. However, S. aureus is an opportunistic “bacterial stalker” that lingers within mammalian populations. Depending upon the strain plus surrounding circumstances, this pathogen exploits an afforded opportunity (i.e. skin break, immunosuppressive state, pre-existing infection, penetrative wound, etc.) via numerous virulence factors that promote survival during the disease process, and then subsequent dissemination to a new host [4]. The focus of this review is upon one group of S. aureus virulence factors, the staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin-1 (TSST-1). These protein toxins abnormally stimulate, and then critically cripple, an immune response of a colonized host by functionally knocking out specific T-cell subsets [5,6]. Unlike many bacterial toxins that directly harm host cells (i.e. puncture membranes, shut down protein synthesis, destroy actin-based cytoskeleton, etc.), the SEs and TSST-1 indirectly affect a host by triggering abnormal immune responses.

Why be concerned with S. aureus and produced superantigens like the SEs and TSST-1? As is alarming evident throughout the microbial world, there is also increasing resistance of pathogenic S. aureus to antibiotics like methicillin (a beta-lactam that prevents cross-linking of cell-wall based peptidoglycan) and vancomycin (a glycopeptide that inhibits peptidoglycan synthesis) [7]. The bacterium is linked to diverse diseases, of which many can become life threatening for anyone regardless of age and health status. Patients colonized by antibiotic-resistant S. aureus (community or hospital acquired) stay in the hospital longer and experience higher morbidity / mortality rates versus those infected with antibiotic-susceptible strains [8]. This sobering fact, linked to medical economics, is further reinforced by data from various countries. An estimated 100 million dollars are spent each year for managing antibiotic-resistant S. aureus in Canadian hospitals [9], in which there was a ten-fold increase in cases from 1995 to 2004. Medical costs are even greater in other countries including the United States, where an estimated 14 billion dollars were spent in 2003 to counter S. aureus infections [10]. There is no evidence suggesting that these numbers will decrease in the near, or distant, future and we simply have few additional tools for effectively fighting S. aureus at this time. S. aureus represents a real health and economic concern throughout our society, today and tomorrow [11-13]. It is an understatement to say that we also need to alter our medical mindset to keep better pace with S. aureus. So, how do we deal more effectively with pathogenic S. aureus now? Perhaps this can be accomplished by employing novel countermeasures (i.e. efficacious vaccines and immunotherapeutics) in conjunction with modern infection control plans regularly practiced in medical facilities.

A basic understanding of how a pathogen survives, flourishes, and evolves can logically lead to alternative means of control. As with many bacterial pathogens, protein toxins
play a major role in various diseases of plants, animals, and humans. At the molecular level, and among diverse bacterial genera, evolution has led to a vast array of biologically similar (and also rather distinct) proteins commonly called toxins [14]. Targeting of these virulence factors for diagnosis, therapy, and vaccines makes good sense into the foreseeable future. *S. aureus* is rather industrious and a single strain can produce multiple SEs (types A-U) that cause one of the most prevalent forms of food poisoning throughout the world [15-17]. Typically, SE intoxication occurs after ingestion of processed meats or dairy products contaminated with *S. aureus* by improper handling and subsequent storage at elevated temperatures. Once carelessly seeded into an accommodating medium, *S. aureus* grows and produces one or more SEs in the form of a soluble toxin 

Toxic shock syndrome caused by TSST-1 of *S. aureus* was first described in 1978 by Todd et al. [24], and later linked to menstruation plus use of highly-absorbent tampons [25-27]. Although TSST-1 was erroneously reported as an enterotoxin (SEF) [28], homogeneous TSST-1 lacks enterotoxicity [29]. TSST-1 intoxication, referred to as toxic shock syndrome (TSS), consists of elevated serum levels of proinflammatory cytokines, rash, hypotension, fever, and multorgan dysfunction [30]. Nonmenstrual forms of TSS in men, women, and children are also attributed to other SEs following *S. aureus* growth upon other body sites and in the bloodstream [31-33]. Recurring bouts of TSS can be common unless the offending strain of *S. aureus* is eliminated or kept at minimal growth. Toxin-specific antibodies play an important role in susceptibility to TSST-1-induced TSS [34], thus a lack of specific immunoglobulins can portend susceptibility to recurring bouts [35,36]. In addition to toxin-specific antibodies, individuals with antibodies against other *S. aureus* antigens are not as readily infected by *S. aureus* versus those with less robust immunity [37]. It is our opinion that experimental vaccines targeting SEs or TSST-1 could be especially useful among high-risk populations that suffer recurring bouts of *S. aureus*–induced disease.

The term "superantigen" was first proposed by Marrack and Kappler [38] to describe microbial proteins that activate a large population of specific T-cells, in contrast to activation by conventional antigens. Superantigens are rather prevalent throughout Nature and produced by various bacterial, viral, and fungal pathogens that cause very diverse diseases [39-53] (Table 1). Such proteins are apparently “successful” virulence factors worthy of perpetuation, at least from a pathogen’s perspective and evolution. Superantigens also differ from conventional antigens in that they bind outside the peptide-binding groove of major histocompatibility complex (MHC) class II, and exert biological effects without internalization or proteolytic processing by antigen-processing cells [38]. Evidently very few MHC class II molecules must be occupied for initiating a superantigen-induced cascade involving T-cell stimulation, proinflammatory cytokine release, etc. [54]. Recognition of a superantigen by T-cell receptor (TCR) typically depends upon the variable region of a β chain (VB), and not Vα and Vβ combinations of TCR used by conventional antigens. Superantigen-induced activation of the host’s immune system elicits abnormally high levels of cytokines and chemokines, enhances expression / activation of cell-adhesion molecules, increases T-cell proliferation, and leads to T-cell apoptosis / anergy (Fig. 1). Sometimes though, reported superantigens prove not to be so as evidenced by *Clostridium perfringens* enterotoxin [55-57].

<table>
<thead>
<tr>
<th>Table 1. Microorganisms that Reportedly Produce Superantigens</th>
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<tr>
<td><strong>Bacteria (superantigen)</strong></td>
</tr>
<tr>
<td>Mycobacterium tuberculosis (MTS)</td>
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<tr>
<td>Mycoplasma arthritidis (MAM)</td>
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<tr>
<td>Staphylococcus aureus (SEA-SEU, TSST-1)</td>
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<tr>
<td>Streptococcus dysgalactiae (SPEGG)</td>
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<td>Streptococcus pyogenes (SPEA, C, G, H, I, J)</td>
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<tr>
<td>Yersinia enterolocitica (YES)</td>
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<td>Yersinia pseudotuberculosis (YPMa, YPMb, YPMc)</td>
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<tr>
<td><strong>Viruses</strong></td>
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<tr>
<td>Cytomegalovirus (?)</td>
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<tr>
<td>Herpes Virus (M1)</td>
</tr>
<tr>
<td>Mouse Mammary Tumor Virus (?)</td>
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<tr>
<td>Rabies Virus (Nucleocapsid)</td>
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<tr>
<td><strong>Fungi</strong></td>
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<td>Candida albicans (Int1).</td>
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According to [39,40], the term “superantigen” was first proposed by Marrack and Kappler [38] to describe microbial proteins that activate a large population of specific T-cells, in contrast to activation by conventional antigens. Superantigens are rather prevalent throughout Nature and produced by various bacterial, viral, and fungal pathogens that cause very diverse diseases [39-53] (Table 1). Such proteins are apparently “successful” virulence factors worthy of perpetuation, at least from a pathogen’s perspective and evolution. Superantigens also differ from conventional antigens in that they bind outside the peptide-binding groove of major histocompatibility complex (MHC) class II, and exert biological effects without internalization or proteolytic processing by antigen-processing cells [38]. Evidently very few MHC class II molecules must be occupied for initiating a superantigen-induced cascade involving T-cell stimulation, proinflammatory cytokine release, etc. [54]. Recognition of a superantigen by T-cell receptor (TCR) typically depends upon the variable region of a β chain (VB), and not Vα and Vβ combinations of TCR used by conventional antigens. Superantigen-induced activation of the host’s immune system elicits abnormally high levels of cytokines and chemokines, enhances expression / activation of cell-adhesion molecules, increases T-cell proliferation, and leads to T-cell apoptosis / anergy (Fig. 1). Sometimes though, reported superantigens prove not to be so as evidenced by *Clostridium perfringens* enterotoxin [55-57].
Although SE or TSST-1 induced apoptosis / anergy leading to compromised immunity is quite complex, the ill effects of intoxication may be linked to diminished L-selectin on specific Vβ-bearing T-cells within one hour of toxin exposure. The end result is decreased signal transduction within the immune system [58,59]. L-selectin (also known as CD62L) binds to carbohydrates on endothelial cells and acts as a homing receptor for T-cells targeting sites of inflammation. Others have discovered that surface levels of TCR-CD3 rapidly decrease within thirty minutes among Vβ-reactive T-cells following SE exposure [60]. The hyperactivation and subsequent proliferation of T cells is transient after encountering SEB [61]. Following an injection of SEB into mice, Vβ8+ T-cells become functionally deleted and no longer respond to the toxin in vitro [62]. Although these anergic cells synthesize less interleukin 2 (IL-2), a cytokine intimately linked to T-cell proliferation, secreted interferon gamma (IFNγ) can still mediate toxic shock after another SEB dose [63]. In addition to IFNγ, an anti-inflammatory cytokine like IL-10 protects the host against SE-induced shock and is produced by SEB-primed T-cells after initial release of proinflammatory cytokines. Production of IL-10 following SE exposure perhaps reflects the host’s attempt (albeit feeble during severe bouts of intoxication!) to counter proinflammatory effects elicited by IFNγ and other cytokines.

2. SES AND TSST-1: NOMENCLATURE AND STRUCTURE

The SEs and related TSST-1 are 22-30 kD proteins (single chain) encoded by plasmids, bacteriophages, or mobile genetic elements and synthesized in vitro by S. aureus during late logarithmic - stationary phases of growth [64]. For decades, SEA-SEE were the only S. aureus enterotoxins described in the literature by relatively few laboratories from around the world. The advent of molecular biology, increasing antibiotic resistance evident among global isolates, recent biotechnology interest (i.e. clinical trials) involving vaccines and immunotherapeutics targeting S. aureus, as well as heightened concerns targeting the SEs (particularly SEB) as a bioterror weapon have attracted more investigators into the field and thus fueled discovery of new SEs. These staphylococcal toxins are basically divided into five homology groups via amino acid sequences, as shown in Table 2 and according to Uchiyama et al. [1].

To eliminate confusion within the literature, in 2004 an international nomenclature committee proposed that any new “staphylococcal enterotoxin” must be verified for emetic activity in a classic, non-human primate assay involving oral administration [65]. This is so even for molecules that share a high level of amino acid homology with other known SEs.
Although this is in principle a good decision, it indeed makes for an interesting scenario as few laboratories have the capability (and monies) to do non-human primate work. Therefore, recent literature contains untested or non-emetic molecules from *S. aureus* with the designation of “staphylococcal enterotoxin-like” (SEL) protein [66]. Some of these molecules lack superantigenicity *in vitro*, yet they are produced during *S. aureus* infection of humans as evidenced by a specific, antibody-mediated immune response [67]. As per committee recommendation, newly discovered toxins possessing more than ninety percent sequence identity (amino acid level) with existing SEs will be designated as a numbered subtype (i.e. SEC1, SEC2, SEC3, etc.). Additionally, as there are only twenty six letters in the alphabet, the SEs discovered after SEZ will be sequentially designated as SE27, SE28, etc. To add yet more complexity, it is not surprising that enterotoxins or SEL proteins produced by other staphylococcal species (i.e. *intermedius*) have also been described in the literature [65]. These molecules should have unique designations from *S. aureus*-derived proteins, such as S(int)EC for a SEC variant synthesized by *Staphylococcus intermedius*. Although there are no easy answers for this burgeoning issue of SE nomenclature, the situation will no doubt become more cumbersome as more SEL proteins are discovered hereafter. It is critical, especially for future researchers, that clarity is carefully placed now upon the taxonomy of these staphylococcal toxins.

Table 2. Amino Acid Sequence-based Groupings of SEs and TSST-1

| Group 1 – SEA, SED, SEE, SEJ, SEN, SEO, SEP |
| Group 2 – SEB, SEC, SEG, SELR, SEU |
| Group 3 – SEI, SEK, SEL, SEM, SEQ |
| Group 4 – TSST-1 |
| Group 5 – SEH |

X-ray crystallographic analyses of the SEs and TSST-1 reveal a common, two domain structure separated by a shallow groove containing both β-sheets and α-helices [68-72]. Examples of this protein architecture for SEA and TSST-1, based upon existing crystallography data, are shown in Fig. (2). Another indicator that the SEs share similar structures is evident by cross-reactivity and neutralization with antibodies [73-77]. Historically, when there were relatively few SEs (i.e. SEA-SEE) recognized in the literature, the SEs were considered serologically distinct entities as per immunodiffusion (i.e. Ouchterlony) assays with polyclonal antisera. Subsequent studies employing more sensitive ELISA technology with polyclonal and monoclonal antibodies reveal that common epitopes do indeed exist between these toxins. The use of antibodies, from a vaccine or immunotherapeutic perspective, will be presented as viable options for fighting severe cases of *S. aureus* infection and ensuing intoxication.

### 3. BINDING OF SES AND TSST-1 TO TARGET CELLS

The staphylococcal superantigens bind to conserved elements on MHC class II with $10^{-8}$ - $10^{-6}$ M affinity, depending upon the MHC isotype and use of either whole cells or purified receptor [78-81]. Generally, human leukocyte antigen (HLA)-DR binds SEs and TSST-1 better than HLA-DP or -DQ, and murine IE acts as a superior receptor than IA. Interactions of SEs and TSST-1 are generally better with human, versus mouse, class II molecules. Of the known staphylococcal superantigens, SEA has the highest affinity for HLA-DR and contains two binding sites [82-86]. The higher affinity site on SEA is located within the C-terminus, which binds to the HLA-DRβ chain in a Zn$^{2+}$-dependent manner. His81 of DRβ coordinates Zn$^{2+}$ with three residues from SEA (His187, His226, and Asp227). This same Zn$^{2+}$-binding motif is also present in SED, as well as SEE, and coordination of Zn$^{2+}$ by the SEA subfamily (SEA, SED, SEE) provides very efficient binding to MHC class II. The second binding

Fig. (2). Crystal structures showing structural similarities of SEA [212] at 1.9 Å resolution and TSST-1 [213] at 2-3 Å resolution. Figures derived from data provided by Entrez’s 3-D database and software for molecular modeling [214].
site on SEA is of lower affinity and located within the N-terminus (Phe47), which in turn interacts with Gln18 found on the α chain of HLA-DR. SEA cooperatively binds as a dimer to HLA-DR, thus cross-linking two MHC class II molecules necessary for cytokine expression [87]. Dimerization of other staphylococcal superantigens, like SEB or SECs, as well as that for MHC class II molecules can play an important role in biological activity in vivo [88,89]. Evidently there are few conformational changes that occur within SEB, or the class II molecule, after forming a heteromeric complex [88].

The N-termini of SEB and TSST-1 have also been identified as MHC class II binding sites via studies with toxin mutants and monoclonal antibodies [71,72,90]. Crystal structures of SEB or TSST-1 complexed with HLA-DR1 definitively reveal different binding mechanisms, although these toxins share the same contact residues on the α chain of MHC class II [88,91]. SEB binds exclusively to the α chain of HLA-DR1 and is not affected by associated peptide, unlike TSST-1 which interacts with the α and β chains of HLA-DR1 or murine IA, as well as the C-terminus of certain bound peptides.

Further examination of SEB - HLA-DR1 co-crystals reveals that nineteen SEB residues and twenty-one residues of HLA-DR1 are intimately involved in complex formation [88] (Fig. 3). Some of these interactions are hydrophobic and occur via Phe44, Leu45, and Phe47 of SEB with HLA-DR1 residues Tyr13, Met36, Ala37, Leu60, Iso63, and Ala64.

Fig. (3). Crystal structure of SEB complexed with HLA-DR1 (2.7 Å resolution) [88]. Data derived from the Protein Data Bank of the Research Collaboratory for Structural Bioinformatics using PyMOL (DeLano Scientific LLC) for molecular modeling. Panels A and B represent different orientations of the SEB - HLA-DR1 complex to better expose residues involved in binding. White-numbered residues are from SEB and black-numbered residues are from HLA-DR1.
Such hydrophobic-based bonds are lost with TSST-1, which contains a Ser instead of the Phe44 in SEB. SEB (Glu67) forms a salt bridge with HLA-DR1 α chain (Lys39), while SEB Tyr89 and Tyr115 provide critical hydrogen bonding to receptor. This interaction is also absent for TSST-1 with HLA-DR1, as the toxin has residue substitutions preventing effective interactions with Lys39 of the α chain. HLA-DR1 residues Tyr13, Gln18, and Lys67 may also bind to Gln43, Phe44, Leu45, and Tyr46 of SEB. Within the SEB disulfide loop (in particular residues Gln92, Tyr94, Phe95, Ser96) there is contact with HLA-DR1 residues 60-68. When complexed, the interface formed between class II and SEB respectively encompasses 780 Å² and 760 Å² which are very similar to typical antigen-antibody (epitope-paratope) combining sites [88].

In addition to MHC class II binding, and like other microbial superantigens, the SEs as well as TSST-1 also interact with the TCR Vβ chain via a groove formed between the two toxin domains [92-95]. Binding of this superantigen-class II complex leads to TCR clustering, subsequent signaling, and potentially detrimental release of proinflammatory cytokines / chemokines from antigen presenting cells (i.e. macrophages plus monocytes) and T cells [96,97]. These toxins each bind a distinct repertoire of Vβ-bearing T-cells, thus revealing a unique biological “fingerprint” useful for confirming a diagnosis of TSS [98,99]. One very unusual exception has been noted, as SEH evidently stimulates T cells in a Vα-specific mode [100]. It is uncertain why SEH is unique from all other SEs tested to date, but this is likely linked to T-cell presentation after the toxin complexes with MHC class II. Additionally, binding of SEH to MHC class II-bearing cells occurs with remarkably high affinity (sub-nanomolar) versus other staphylococcal superantigens [101].

As per different Vβ specificities of the SEs and TSST-1 (Table 3), it is logical that MHC class II / TCR contacts differ for each toxin. As an example of this diversity, mutagenesis of the SECs reveals that a Tyr or Val (residue 26) differentially affects Vβ3 and 13.1 interactions [102]. Val91of SEC2 also impacts binding to TCR, but an equivalent amino acid is not found in either SEA (Tyr94) or SEB (Tyr91). Molecular mutagenesis of SEA into SEE, or SEE into SEA, is interesting from a T-cell stimulation perspective. A comprehensive study with these toxins reveals that only two residues (Ser206 and Asn207) of SEA, when switched to those found in SEE (Pro206 and Asp207) or vice versa, generates a toxin with the Vβ stimulatory profile like the other [103].

Additionally, orientation and binding affinity of superantigen with the α-chain of MHC class II affect TCR Vβ interactions. The binding affinity between TCR and a SE is relatively weak (Kd ~10⁻⁶), but this interaction is strengthened by prior binding of toxin to MHC class II [94]. SEB residues that interface with TCR include solvent-exposed Asn23, Asn 60, Tyr61, and regions of the disulfide loop (Cys93-Cys113) (Fig. 4). A cooperative effect is also observed for SEA with host TCR and MHC class II, ultimately enhancing tricomplex stability that stimulates macrophages, monocytes, and T cells [95]. In the case of TSST-1, T-cell activation may be influenced by contact between the C-terminus of TSST-1 with peptide lying in the antigen-binding groove of HLA-DR [91]. Specifically, histidines 132, 135, and 140 of TSST-1 are important for TCR interactions and subsequent release of proinflammatory cytokines from T cells [104-106].

It is clear that the superantigenic potential of these S. aureus toxins results from a cooperative process between microbial toxin and cell-surface receptors, which ultimately triggers a detrimental response within the host. From a microbiologist’s or biochemist’s perspective, the SEs and TSST-1 are rather complicated protein toxins via their intricate interactions with multiple cell types and different receptors that negatively impact the immune system. The biological effects of SEs and TSST-1 are intimately linked to a myriad of host-derived effectors (i.e. proinflammatory cytokines and chemokines) that, in large enough concentrations, can cause great harm to a host. Normally, such effector molecules are meant to afford host protection towards a plethora of pathogenic invaders encountered on a daily basis. In many ways, a vigilant immune system that defends against life’s little challenges can ironically become one’s demise from within.

4. IN VIVO EFFECTS OF SES AND TSST-1: A MULTIFACTORIAL EVENT

In humans and nonhuman primates, SEs can elicit an emetic response and in rare cases toxic shock following ingestion of very low microgram quantities [15,16]. In contrast, TSST-1 does not cause emesis after ingestion but can

### Table 3. Human Vβ Specificity of Select SEs and TSST-1

<table>
<thead>
<tr>
<th>SEs</th>
<th>Vβ Specificity</th>
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<tbody>
<tr>
<td>SEA</td>
<td>Vβ1, 1.1, 5.1, 5.2, 5.3, 6.3, 6.4, 6.9, 7.2, 7.3, 7.4, 7.9, 8, 9.1, 16, 18, 21.3, 22, 23</td>
</tr>
<tr>
<td>SEB</td>
<td>Vβ3, 6.4, 12, 13.2, 14, 15, 17, 20</td>
</tr>
<tr>
<td>SEC1</td>
<td>Vβ3, 6.4, 6.9, 12, 13.2, 14, 15, 17, 20</td>
</tr>
<tr>
<td>SEC2</td>
<td>Vβ12, 13.1, 13.2, 14, 15, 17, 20</td>
</tr>
<tr>
<td>SED</td>
<td>Vβ1, 5, 6.9, 7.4, 8, 12</td>
</tr>
<tr>
<td>SEE</td>
<td>Vβ3.1, 6.1-6.4, 6.7, 6.9, 8.1, 16, 18, 21.3</td>
</tr>
<tr>
<td>SEG</td>
<td>Vβ3, 12, 13.1, 13.2, 13.6, 14, 15</td>
</tr>
<tr>
<td>SEI</td>
<td>Vβ1.1, 5.1, 5.2, 5.3, 6, 23</td>
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<tr>
<td>SELL</td>
<td>Vβ3.1, 5.2, 6.7, 7, 9, 16, 22</td>
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<tr>
<td>SELM</td>
<td>Vβ3.1, 5.2, 5.3, 6, 18, 21.3, 23</td>
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<tr>
<td>TSST-1</td>
<td>Vβ2, 4</td>
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Data derived from multiple references [1,40,99,215].
evoke systemic shock via growth of toxigenic S. aureus on mucosal surfaces [29,107]. Unlike a number of other entero-
toxins, specific cells and receptors in the intestinal tract have not been unequivocally linked to oral intoxication by a SE. After many decades of research, it is disappointing that we do not have a better understanding of toxin receptor(s), trafficking, and overall intoxication process leading to enteric ill-effects. Much effort indeed has been spent by many laboratories upon the superantigen aspects of these toxins and TSS, which may or may not play an important role in food poisoning.

In regards to enteric effects of the SEs, when SEA or SEB are injected intraperitoneally there is distal induction of Fos (a transcription-linked cell activator) throughout the brain via vagus nerve stimulation from the gut. Such data suggest that the peripheral presence of a SE affects the central nervous system [108,109]. Capsaicin, a small molecular weight (305 daltons) metabolite in hot chili peppers, depletes peptidergic sensory nerve fibers and diminishes SE effects in mammals [110,111]. Similar results (capsaicin-induced inhibition of vagal nerve stimulation) are evident for other gut-originating, protein toxins like E. coli heat stable enterotoxin [112] and Clostridium difficile toxin A [113]. SE communication directly (or indirectly) with the vagus nerve via MHC class II-bearing cells may be linked to fibrous vagus bundles found throughout the abdomen [114]. From a therapeutic perspective, experiments involving the central or peripheral nervous systems and SEs are clearly lacking in the literature.

Various investigators have attempted to locate a specific emetic domain within the SEs, but findings have been lim-
ited and equivocal. Single residue changes in SEA (Leu48Gly) and SEB (Phe44Ser) result in molecules that still elicit emesis, yet do not bind MHC class II or cause T-cell stimulation [115]. The disulfide loop in various SEs, which is absent in TSST-1, may be responsible for the emetic activity of SEs but that too remains controversial between different laboratories [116,117]. Carboxymethylation of histidines on SEA [118] or SEB [119] generates proteins devoid of enterotoxicity, or skin reactivity [120,121], yet these modified toxins still retain superantigenicity. The chemically-modified SEB also inhibits, in a presumed competitive fashion for receptor, the enteric effects of wild-type SEB when concomitantly given orally to nonhuman primates [121]. Lack of enterotoxicity attributed to carboxymethyl-substituted SEA is not due to enhanced degradation by gastric proteases [122], thus suggesting that this modified form of SEA retains a native conformation naturally resistant to proteolysis. Analysis of each histidine and effects upon SEA-induced emesis plus superantigenicity reveals that His61 is important for emesis, but not T-cell proliferation [122]; therefore, demonstrating that an emetic response and superantigenicity represent distinct properties of the toxin. Another group used antibodies that prevent SEA-induced emesis by targeting SEA residues 121-180, a region lacking the disulfide loop (Cys91-Cys105) and histidines [123].

SE-induced stimulation of mast cells and subsequent release of cysteinyl leukotrienes (eicosanoid mediators of inflammation) cause emesis, as well as skin reactions, in primates [124,125]. Murine Vβ8+ T-cells found within intestinal Peyer’s patches are adversely affected by orally-administered SEB, as determined by a subsequent lack of in

![Crystal structure of SEB complexed with Vβ 8.2 of mouse TCR (2.4 Å resolution) [93]. Data derived from the Protein Data Bank of the Research Collaboratory for Structural Bioinformatics using PyMOL (DeLano Scientific LLC) for molecular modeling. The numbered residues in the green region are from SEB.](image-url)
among V<sup>88</sup> mice also cause a dose-related, immunological tolerance to additional infections. Footpad injections of SEB in SCID mice suggests that regulatory T-cells critically participate in SEB-induced inflammation of the intestines [130].

Effects of the SEs upon mucosally-located T cells may also explain earlier results showing that SE ingestion by nonhuman primates yields transient resistance to an even higher dose of the same toxin [131]. However, this effect is not evident when these animals are given another SE serotype orally and perhaps such findings are due to toxin-specific stimulation of unique Vβ-bearing T-cells. In addition to toxin-specific resistance elicited by one oral dose of a SE, chronic intravenous exposure to SEA can functionally delete all Vβ-reactive T-cells in mice [132]. Such striking alterations of the immune system could perhaps induce an immunocompromised state and transiently increase susceptibility to additional infections. Footpad injections of SEB in mice also cause a dose-related, immunological tolerance among Vβ<sup>88</sup> T-cells [133]. Another study in mice given wild-type SEA intranasally reveals resistance to a subsequent lethal challenge (intraperitoneal) with SEA, but not a heterologous superantigen like TSST-1 [134]. A recombinant form of SEA devoid of superantigenicity, when given intranasally, does not afford the same protection towards wild-type SEA. This effect is not due to toxin-specific antibody or depletion of SEA-reactive T-cells, but there is a significant increase in serum levels of IL-10. Previous studies show that IL-10 affords protection against SE-induced effects [135]; however, a recent study of neonatal TSS triggered by TSST-1 shows that neonates with this disease have elevated levels of IL-10 versus individuals without TSS [136]. Overall, existing literature suggests that high circulating levels of IL-10 lead to reduced severity of superantigen-based disease.

Circulation of SEs or TSST-1 from a S. aureus infection site (i.e. intestines, mucosa, skin), or following ingestion of toxin in classic food poisoning, could have more profound effects upon the host versus if the toxin remains localized. Studies employing a human colon monolayer (Caco-2 cells) show transcytosis of SEA, SEB, and TSST-1, while in vivo results (mice) reveal that SEB enters the bloodstream more readily than SEA after ingestion [137]. A more recent study suggests that a superantigen-conserved, dodecapeptide from TSST-1 (Phe119-Asp130) plays a role in epithelium penetration and toxicity in vivo [138]. Overall, such data suggest that these toxins cross the gastric or vaginal mucosa and subsequently circulate throughout the body. In vitro, these toxins are not cytotoxins that directly kill human intestinal cells like Henle 407 [139]. However, incubation of a human colonic monolayer (T84 cells) with SEB and peripheral blood mononuclear cells (PBMC) increases ion flow across the cell-based barrier, thus suggesting indirect toxin effects upon gut mucosa via the immune system and released proinflammatory cytokines [140]. A cytokine protective against various bacterial superantigens in vivo, IL-10 (but not IL-4), dose-dependently inhibits permeability of T84 cell monolayers in vitro when added before or concomitantly with SEB [135,140-142].

As previously discussed, superantigen interactions with MHC class II and selected TCR Vβ enable the SEs and TSST-1 to perturb the immune system towards releasing high concentrations of proinflammatory cytokines. Another result of such perturbations is that the SEs and TSST-1 are pyrogenic in humans, nonhuman primates, ferrets, and rabbits [143-145]. This is largely attributed to elevated levels of proinflammatory cytokines that include synergistic actions of IL-1 and tumor necrosis factor alpha (TNFα), which ultimately induce fever via the hypothalamus [146]. Serum levels of IFNγ, IL-2, and IL-6 also increase after toxin exposure. IFNγ augments immunological responses by increasing the MHC class II levels on antigen presenting, epithelial, and endothelial cells. IFNγ also upregulates expression of TNFα and IL-1 receptors, that when bound by ligand, increases adhesion molecules on endothelial cells as well as promotes leukocyte binding plus recruitment. Shock induced by superantigens ultimately results from cumulative ill-effects of proinflammatory cytokines adversely affecting various organs throughout the host [147].

**Animal Models for SEs and TSST-1: Necessary Steps Toward a Better Understanding**

Mice are often used for obtaining a basic understanding of how toxins (and other biologically-active substances) interact in vivo, which includes the immunological mechanisms that promote superantigen-mediated shock [148-156]. Although these animals curiously lack an emetic response, they are cost-effectively ideal for in vivo screening of potential vaccines and therapeutics. Mice are naturally less susceptible to SEs and TSST-1, versus humans, because of lower affinity for murine MHC class II. Potentiating agents like D-galactosamine, actinomycin D, lipopolysaccharide (LPS, also known as endotoxin), and even viruses (i.e. influenza or lymphocytic choriomeningitis) amplify the toxic effects of superantigens in mice. Many of our studies involve an LPS-potentiated mouse model [104,141,150], as various in vitro and in vivo assays reveal a natural synergy (many log-fold) between SEs / TSST-1 and LPS [157-162]. As little as two micrograms of LPS alone in humans can cause endotoxic shock [163], thus only picogram quantities of endotoxin in conjunction with a superantigen can cause quite severe, life-threatening effects [164]. Upon considering the many Gram-negative bacteria that compose the intestinal flora, and increased numbers found in the vaginal flora of TSS patients, odds of this synergy naturally occurring are really quite high [164,165]. In concordance with human data, there is a good correlation between increased serum levels of IL-1, IL-2, TNFα, and/or IFNγ with SEA-, SEB-, or TSST-1-induced shock in mice [148-160]. These efforts coincide with others involving SEA and genetic knockout mice deficient in IFNγ or the p55 receptor for TNFα [141]. In addition to potentiation of SE effects in mice by other compounds, it has been recently reported that C3H/HeJ mice (non-LPS responders) lethally respond to one or two timed doses of SEB (seven
micrograms total) given intranasally [166]. IL-2 and monocyte chemoattractant protein-1 (MCP-1) play a critical role in this particular model.

Transgenic mice expressing human HLA-DQ6 and CD4 succumb to normally sublethal amounts of SEB (i.e. D-galactosamine potentiation in wild-type animals), with serum levels of TNFα correlating with lethal shock [154]. Transgenic mice expressing human HLA-DR3 and CD4 lethally respond to SEs without a potentiating agent, perhaps affirming yet another model for future in vivo studies [155]. Fewer parameters (i.e. potentiating components) used in a model enable easier interpretation of results, especially for therapeutic-based studies in vivo. When SEB is incubated with PBMC from HLA-DR3/CD4 mice, more IL-6 and IFNγ are released relative to similarly-treated PBMC from non-transgenic controls. These data suggest that proinflammatory cytokines, like those elevated in other animals or humans, also play an important role during SE-induced shock in transgenic mouse models.

In addition to lethality as an endpoint for SE or TSST-1 intoxication, temperature is a readily measured parameter of shock-induced illness. Historically, rabbits afford an attractive model for SE- and TSST-1-induced shock with either temperature or lethal endpoints [162,167,168]. Similar to humans with TSS [157], rabbits exposed to TSST-1 or SEB have subsequently heightened levels of LPS in the bloodstream [169,170]. Increased levels of circulating LPS may be due to toxin-elicited impairment of liver function [162,171]. Goats have also been used for studying the in vivo effects of TSST-1 and SEB, involving temperature fluctuations following intravenous administration [172]. Temperature is also readily measured with LPS-potentiated SE and TSST-1 in mice (BALB/c or C57BL/6) implanted with a subcutaneous transponder [141] or telemetry device [173]. In such non-lethal models, mice experience a significant decrease in temperature (there was never any detectable increase) within ten hours of intoxication. Additionally, C3H/HeJ mice that are dual-dosed intranasally with SEB also experience measurable temperature fluctuations [166].

Finally, an unusual animal model involves ferrets given SEB orally, with end results being emesis and rapid fever [145]. Milligram quantities of toxin are however necessary for an effect versus microgram amounts commonly used in mice, rabbits, or nonhuman primates. In addition to potential availability issues of these unique laboratory animals, the ferret model seems less feasible than others for vaccine or therapeutic discovery based upon toxin amounts needed for a biological effect.

5. SUPERANTIGEN TRICKERY: A SUBVERSIVE PUSH TOWARDS AUTOIMMUNITY

As superantigens specifically activate Vβ-bearing T cells, some that are normally quiescent and autoreactive, the danger of host autoimmunity is real. Arthritis, psoriasis, atopic dermatitis, collagen vascular disease, as well as inflammatory bowel disease have all been linked (to varying degrees) with microbial superantigens like the SEs and TSST-1 [127-129,174-179]. Although many proinflammatory cytokines in high concentrations can be deleterious for the host, IL-10 actually plays a protective role by preserving a self-tolerant state within the host via downregulating macrophage and monocyte functions [180].

When *S. aureus* colonizes skin, a toxic byproduct of growth (i.e. SEB) can readily inflame the human epidermis perhaps by degranulation of cutaneous mast cells [120,181]. Severe atopic dermatitis induces apoptotic T-cells, which can have dire consequences for the host as evidenced by chronic infections and disease severity [182]. However, versus normal controls, a mild case of dermatitis induces hyperreactive T-cells towards SEB which is concomitantly associated with elevated levels of proinflammatory cytokines. Specific IgE against SEs and TSST-1 is also evident in atopic dermatitis patients, but not so in others even when colonized by *S. aureus*, thus suggesting additional linkage of these toxins to this disease [183]. Evidently the density of *S. aureus* on skin correlates with development of atopic dermatitis [184]; therefore, multiple factors such as environment, hygiene, resident flora, and antibiotic usage all play important roles in development of this disease. Among cancer patients, radiation therapy may also induce a very severe dermatitis linked to prior colonization by *S. aureus* [185].

6. ANTIBODY-MEDIATED PROTECTION AGAINST SES AND TSST-1: VACCINES PLUS ANTIBODY-BASED THERAPEUTICS

To date, there is remarkably no Food and Drug Administration (FDA)-approved therapeutic (in addition to commonly-used antibiotics) or vaccine to counter *S. aureus*. This is a particularly pressing issue as per increased isolation of toxin-producing, antibiotic-resistant strains of *S. aureus* among select (and very susceptible!) populations, that include: 1) dialysis, trauma, intensive care, as well as immunocompromised patients; 2) patients with surgical implants and particularly ventriculoperitoneal shunts; 3) nursing home residents; 4) diabetics; 5) healthcare providers; 6) military personnel; and 7) premature babies [186,187]. Veterinary vaccines for *S. aureus* have been developed that afford reasonable protection against mastitis in cattle, but the use of either whole bacteria (formalin killed and chemically-mutagenized) or crude cell lysates as inoculum for humans is rather remote [188-190].

Regarding vaccines for humans against *S. aureus*, an experimental product called StaphVAX® targets capsular polysaccharide serotypes 5 and 8 (found on many human plus veterinary clinical isolates) and has been thoroughly tested through phase III clinical trials [191]; however, this vaccine ultimately proved less than efficacious [190]. StaphVAX® is composed of *S. aureus* polysaccharides conjugated to *Pseudomonas aeruginosa* exotoxin A (recombinantly detoxified form). Evidently further studies are planned to include other antigens with this product, such as the cell-surface polysaccharide 336, alpha toxin, and Panton-Valentine leukocidin. It is possible that surface-targeting antibodies are not efficacious because the bacterium may survive in phagocytes even after binding of opsonizing antibodies [190]. Additionally, capsular antigens (serotypes 5 and 8) are present during stationary, but not growth, phases of *S. aureus in vitro*. It is uncertain if this is also the case during an infection, but if so,
perhaps it would be better from a vaccine perspective to target antigens expressed during the early growth phase of \textit{S. aureus}. For reasons not totally understood, the capsular polysaccharide-based vaccine for \textit{S. aureus} falls short of similar-based vaccines used successfully against other bacterial pathogens like \textit{Haemophilus influenzae} and \textit{Streptococcus pneumoniae}.

To neutralize other \textit{S. aureus} virulence factors like the SEs and TSST-1, there are at least three important targets: (1) TCR - toxin - MHC class II interactions; (2) accessory, co-stimulatory, or adhesion molecules involved in activation and effector functions of T-cells; and (3) cytokine release by activated T-cells as well as macrophages. Inhibition of all, or just one, of these three targets has been reported both in \textit{vivo} and \textit{in vitro}, thus representing viable strategies towards curbing the biological effects of these bacterial toxins.

Various groups have developed experimental vaccines for the SEs and TSST-1. This seems like a wise strategy, as preexisting antibodies against SEs and TSST-1 play an important role in disease outcome [34-36] and the use of intravenous immunoglobulins (IVIG) has proven efficacious in humans after the onset of staphylococcal or streptococcal venous immunoglobulins (IVIG) has proven efficacious in humans after the onset of staphylococcal or streptococcal toxic shock [192,193]. Given this wealth of information, it is logical that vaccination spurring a targeted humoral response might be useful for preventing TSS caused by SEs and TSST-1. Recombinant-attenuated SEA, SEB, and TSST-1 not binding to MHC class II and/or specific V\beta-bearing TCR molecules represent experimentally successful vaccines preventing toxic shock in different animal models [194-198]. These vaccines, when given either parenterally or mucosally, have proven efficacious against a toxin challenge or \textit{S. aureus} infection in various animal models. Other murine and nonhuman primate studies with formalin-inactivated SEB report protection towards a homologous toxin challenge following parenterally-, or mucosally-, administered vaccine [199,200]. However, subsequent studies have demonstrated that formaldehyde treatment of proteins adversely affects processing and subsequent presentation to the immune system [201], especially if the modified protein represents a mucosal immunogen [202].

From a vaccine perspective, antigens other than the SEs and TSST-1 may be important for mitigating various diseases caused by \textit{S. aureus}. In the end, vaccines containing a few select virulence factors possessing diverse biological activities will likely be most efficacious. There are multiple examples of dissimilar \textit{S. aureus} antigens efficacious against the bacterium in various animal models, when used as a sole target antigen, such as: 1) iron surface determinant B which is a conserved iron-sequestering protein on the cell surface [203]; 2) recombinantly-attenuated alpha toxin unable to form pores in cell membranes [204]; 3) glutathione S transferase - TSST-1 fusion using a TSST-1 molecule incapable of binding TCR [205]; 4) staphylococcal-conserved, glyceraldehyde-3-phosphate dehydrogenase homologs [206]; and 5) a unique vaccine consisting of an adsorbed protein from \textit{Candida} that structurally mimics a fibrinogen / fibrin binding protein (clumping factor A) of \textit{S. aureus} [207]. Protection afforded by the latter vaccine is more T-, versus B-, cell mediated while a humanized monoclonal antibody targeting \textit{S. aureus} clumping factor A is readily tolerated by patients and preliminary results show efficacy [208]. Even though these various studies provide hope, there is still much more work to be done towards generating an efficacious, FDA-approved vaccine against \textit{S. aureus}. A reduction in nasal carriers of \textit{S. aureus} would logically diminish bacterial spread throughout a population, thus a vaccine that stimulates mucosal immunity might be more beneficial versus that predominantly activating a systemic immune response.

Besides active vaccination against \textit{S. aureus}, the use of immunotherapeutics is particularly appealing for the medical community. To address these needs there have been to date three human antibody products used in clinical trials; however, none has received FDA approval for various reasons [190]. These reagents vary in targeted antigens, and include: 1) AltaStaph\textsuperscript{®} – human polyclonal antiserum (following vaccination with aforementioned Staph VAX\textsuperscript{®}) against \textit{S. aureus} capsular polysaccharides 5 and 8; 2) Veronate\textsuperscript{®} (also known as INH-A21) – enriched antibodies from human sera targeting staphylococcal adhesins like clumping factor A; and 3) Pagibaximab\textsuperscript{®} – humanized mouse antibody (monoclonal) targeting lipoteichoic acid, a cell wall / membrane constituent of \textit{S. aureus}. In a non-commercial venture, LeClaire et al. [209] described the protective effects of chicken immunoglobulin Y generated against SEB in a nonhuman primate model. Passive transfer of this antitoxin up to four hours after SEB exposure proved quite effective. An advantage of this antibody type, versus those derived from other nonhuman species, is less reactogenicity in humans.

In summary, there is a growing demand for efficacious vaccine- and therapeutic-based antibodies targeting \textit{S. aureus}. Increasing antibiotic resistance of \textit{S. aureus} isolated from around the world, and a decreasing ability to effectively fight these microbes in clinical settings, are major driving forces for this emerging market. A successful product would likely block attachment of \textit{S. aureus} to host tissue, neutralize multiple toxins, and/or enhance both cell-mediated plus humoral immunity.

7. CONCLUDING REMARKS

\textit{S. aureus} is a formidable pathogen producing various protein superantigens \textit{(i.e.} SEs plus TSST-1\textit{)} that interact with MHC class II and TCR molecules on host cells. Such binding events subsequently cause the immune system to over react through hyperproduction of various immunomodulators like proinflammatory cytokines and chemokines. Amino acid homologies, as well as similar conformations plus biological activities, among the SEs and TSST-1 suggest common evolutionary paths. Superantigens produced by other bacteria, viruses, and even fungi further imply that these proteins afford a survival advantage that perhaps includes delayed clearance from a host [210,211]. To genetically conserve and then expend valuable energy for synthesizing superantigens from one generation to the next implies a biological success within the microbial world. Perhaps there are other activities attributed to these molecules that have not yet been defined, as per the limits of existing assays commonly used in research laboratories today?

Hyperstimulation of T-cells, release of unhealthy levels of cytokines plus chemokines, and subsequent immunosup-
pression are all induced by the SEs or TSST-1. Interference with any of these steps should prevent, or at least mitigate, toxic manifestations of these staphylococcal superantigens and thus decrease morbidity and mortality attributed to S. aureus infections. Antibody-based interventions targeting the SEs and TSST-1 could occur via various aforementioned vaccines and/or immunotherapeutics. To date, such medical reagents are only experimental but some day will hopefully become clinically-viable options for physicians.

To counter S. aureus, it is likely that targeted virulence factors in addition to the SEs and TSST-1 will be useful. Clearly, as a society, we must take more aggressive action towards countering S. aureus as evidenced by the pathogen’s increasing antibiotic resistance, role in multiple diseases, and established prevalence throughout the world. We believe vaccine and immunotherapeutic attempts, although experimental, are positive steps in the proper direction. Various clinical trials focused upon clearance of pathogenic S. aureus from humans, with less than stellar results, perhaps suggest we need to expand our approaches to more than one or two virulence factors. By medically attacking multiple S. aureus targets there will likely be better results with, or without, the use of antibiotics whose efficacy is being eroded at an alarming rate. Furthermore, S. aureus is just one of many pathogens that we are slowly losing our ability to control with current medicinal tools. It really is time to think, and then do, outside of the existing medical box we have now painted ourselves into as we rapidly end this first decade of the 21st Century.

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CONFLICTS OF INTEREST

The authors report no conflicts of interest, and all expressed opinions are those solely of the authors and do not reflect in any way the official views of the United States government.

ABBREVIATIONS

IVIG = intravenous immunoglobulin
IFNγ = interferon gamma
IL = interleukin

LPS = lipopolysaccharide
MHC class II = major histocompatibility complex class II
PBMC = peripheral blood mononuclear cells
SE = staphylococcal enterotoxin
TSST-1 = toxic shock syndrome toxin-1
TCR = T-cell receptor
TNF = tumor necrosis factor

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Staphylococcus aureus Superantigens


Staphylococcus aureus Superantigens


