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ARTICLE *in* IMMUNOLOGIC RESEARCH · MAY 2014

Impact Factor: 3.1 · DOI: 10.1007/s12026-014-8538-8 · Source: PubMed

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DOI 10.1007/s12026-014-8538-8

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Staphylococcal toxic shock syndrome: superantigen-mediated enhancement of endotoxin shock and adaptive immune suppression

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Abstract Infectious diseases caused by *Staphylococcus aureus* present a significant clinical and public health problem. *S. aureus* causes some of the most severe hospital-associated and community-acquired illnesses. Specifically, it is the leading cause of infective endocarditis and osteomyelitis, and the second leading cause of sepsis in the USA. While pathogenesis of *S. aureus* infections is at the center of current research, many questions remain about the mechanisms underlying staphylococcal toxic shock syndrome (TSS) and associated adaptive immune suppression. Both conditions are mediated by staphylococcal superantigens (SAgs)—secreted staphylococcal toxins that are major *S. aureus* virulence factors. Toxic shock syndrome toxin-1 (TSST-1) is the SAg responsible for almost all menstrual TSS cases in the USA. TSST-1, staphylococcal enterotoxin B and C are also responsible for most cases of non-menstrual TSS. While SAgs mediate all of the hallmark features of TSS, such as fever, rash, hypotension, and multi-organ dysfunction, they are also capable of enhancing the toxic effects of endogenous endotoxin. This interaction appears to be critical in mediating the severity of TSS and related mortality. In addition, interaction between SAgs and the host immune system has been recognized to result in a unique form of adaptive immune suppression, contributing to poor outcomes of *S. aureus* infections. Utilizing rabbit models of *S. aureus* infective endocarditis, pneumonia and sepsis, and molecular genetics techniques, we aim to elucidate the mechanisms of SAg and endotoxin synergism in the pathogenesis of TSS, and examine the cellular and molecular mechanisms underlying SAg-mediated immune dysfunction.

Keywords Staphylococcal toxic shock syndrome · Superantigens · TSST-1 · *Staphylococcus aureus*

Staphylococcus aureus diseases affect ~500,000 individuals each year in the USA [1]. Currently, *S. aureus* is the leading cause of infective endocarditis (accounting for ~40,000 cases/year) [1–4] and osteomyelitis, and the second leading cause of sepsis [1, 5, 6]. *S. aureus* also accounts for ~70,000 cases of pneumonia [1, 7], more than 120,000 postsurgical infections [1, 8], and ~6,000 cases of menstrual toxic shock syndrome each year in the USA [7, 9]. Contributing to these infections are both methicillin-susceptible and -resistant *S. aureus*. Currently, there is little understanding of *S. aureus'* mechanism of pathogenesis and the nature of protective immunity against infection. Vaccination and immune therapy development against *S. aureus* has proven to be challenging in times when there is an increasing prevalence of infections caused by multidrug-resistant strains and an increasing rate of both healthcare- and community-associated infections. *S. aureus* pathogenicity is mediated by numerous virulence factors, among which the staphylococcal superantigens (SAgs) play a key role [5].

Staphylococcal enterotoxins (SEs) and staphylococcal enterotoxin-like (SE*l*s) molecules, collectively known as SAgs, are major secreted virulence factors of *S. aureus*. Almost every *S. aureus* strain encodes for and can variably produce SAgs when the opportunity arises [5]. SAgs have previously been known to cause vomiting and diarrhea (hence the name enterotoxins) [10], but are also highly

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Published online: 11 May 2014

lethal in humans [11]. Over the past years, SAgs have been shown to be critical contributors to the pathophysiology of *S. aureus'* life-threatening infections, including sepsis, infective endocarditis, and necrotizing and hemorrhagic pneumonias [12]. Most of these diseases have high mortality rates, even with currently available treatment strategies. Lethality is critically dependent on development of toxic shock syndrome (TSS) and progression to toxic shock. TSS is mediated by a complex interaction of SAgs with the host resulting in extensive immune dysregulation and multi-organ dysfunction [7].

Humans are exquisitely sensitive to SAgs, where intravenous doses as low as 0.001 μg/kg lead to fever and hypotension (hallmark symptoms of TSS) [11]. Similarly, in rabbits, intrapulmonary inoculation of the SAg TSST-1 or continuous exposure at 0.05 μg/kg implanted subcutaneously in miniosmotic pumps causes death in all treated animals [13]. Interestingly, the susceptibility of humans and rabbits to SAgs correlates with the significant presence of gram-negative rods, which produce toxic lipopolysaccharide (LPS), specifically, in humans *Escherichia coli* colonizing the intestinal and vaginal tracts, and in rabbits, intestinal *E. coli* and/or *Pasteurella multocida* colonizing their mucosal membranes [14–16]. Resistance of rodents and non-human primates to SAgs and development of TSS are associated with significantly lower colonization by toxic LPS-producing organisms [17]. One of our major goals is to elucidate the mechanism of LPS and SAg synergism during TSS in in vitro and in vivo systems. A second goal is to elucidate the cellular and molecular mechanisms of immune suppression mediated by *S. aureus* SAgs. Clinical evidence suggests that superantigenicity (massive activation of macrophages and $CD4^+$ T cells and the resultant cytokine storm) affects adaptive immune cells in a way that interferes with their ability to mount *S. aureus*-specific responses. This aspect of *S. aureus* pathogenesis is grossly understudied yet, it is critical for the successful development of vaccine strategies and immunotherapies.

Superantigen enhancement of endotoxin shock

Toxic shock syndrome is an acute, life-threatening condition associated with infections by *S. aureus* or *Streptococcus pyogenes* in susceptible individuals. Clinically, the syndrome is characterized by fever, rash, hypotension, desquamation of the skin upon recovery, and involvement of three or more of the following organ systems: gastrointestinal, renal, hepatic, central nervous system, mucous membranes, muscular, and hematologic [18]. Historically, TSS has been associated with the use of super-absorbent tampons; however, other risk factors of TSS development include colonization of mucous membrane surfaces with toxin-producing *S. aureus* strains, breaks in the skin, surgical procedures, and other predisposing conditions; thus, the population at risk includes all—women, men, and children [19–22]. While all staphylococcal enterotoxins (if produced at sufficiently high levels) can cause TSS, TSST-1 causes nearly all cases of menstrual TSS and at least half of non-menstrual TSS (TSS associated with *S. aureus* infections at various body sites) [7, 23, 24]. Staphylococcal enterotoxin B and C (SEB and SEC) account for the other half of non-menstrual staphylococcal TSS cases [7, 24].

TSST-1 was identified and characterized in 1979 [25] and confirmed as the etiologic agent of menstrual TSS in 1981 [26]. The defining functional properties of TSST-1 (and all SAgs) are pyrogenicity (ability to induce fever), non-cognate antigen T-cell activation, and enhancement of susceptibility to endotoxin shock [7, 27]. While induction of fever and non-cognate antigen T-cell activation are well-characterized effects of SAgs, many questions remain regarding the mechanism of SAg and LPS synergism in mediating the severe course and outcomes during TSS [7, 27]. LPS is a bacterial endotoxin produced primarily by *E. coli,* which is part of the gut flora of both humans and, to a smaller degree, rabbits, and *P. multocida,* an opportunistic pathogen in rabbits [15, 16, 28, 29]. Early reports of staphylococcal enterotoxin enhancement of host susceptibility to LPS dates back to 1964 when Sugiyama et al. [30] described SEA- and SEB-mediated enhancement of endotoxin lethality. Subsequently, Schlievert et al. identified and characterized TSST-1 (previously designated as staphylococcal pyrogenic exotoxin C) and demonstrated, for the first time, its capacity to synergize with LPS in producing LPS-mediated shock, and myocardial and liver damage [26, 31, 32]. While neither of the toxins administered alone was lethal in a rabbit model, both toxins together increased the susceptibility to toxic shock and death by more than 50,000-fold [32].

Rabbits and humans respond similarly to SAgs and LPS, with one major reason being that the severe and lethal outcomes associated with both molecules are critically dependent on their effects on the cardiovascular system, and the cardiovascular physiology of rabbits closely resembles that of humans [13, 33, 34]. SAg enhancement of endotoxin shock is reliably observed in rabbits during normal development. Young adult rabbits succumb to TSST-1 at doses of 50 μg/kg [35] and to LPS at 535 μg/kg [32]. However, in 8-month-old rabbits, *P. multocida* colonization increases, rendering rabbits 1,000 times more susceptible to LPS (LD_{100} 0.5 μg/kg). At the same time, the susceptibility of rabbits to the lethal effect of TSST-1 increases 1,000-fold (LD_{100} 0.05 μg/kg) [7]. Therefore, host susceptibility to the detrimental effects of TSST-1 and LPS increases by up to one million-fold in the presence of

both molecules [32]. This effect is seen with all SAgs tested [32, 36]. At present, SAg enhancement of endotoxin shock is not universally considered a critical contributor of TSS. However, clinical and rabbit experiments support the hypothesis that the ability of TSST-1 and staphylococcal enterotoxins to enhance the susceptibility to endogenous endotoxin is an important mechanism in the etiology of TSS [7, 37, 38].

Under normal conditions, a small amount of endotoxin is absorbed in the intestine and channeled via portal vein to the liver where it undergoes detoxification [39]. TSST-1 may act like other staphylococcal toxins and inhibit RNA synthesis or damage hepatocyte mitochondrial membranes, altering the function of Kupffer cells (the macrophages of the liver) and sinusoidal cells that are crucial for LPS detoxification [40, 41]. As a result, non-metabolized LPS enters the systemic circulation causing systemic endotoxemia and mediating the detrimental effects of endotoxin. In fact, endotoxemia occurs in both humans and rabbits during TSS [42]. Host-derived endotoxin was confirmed as the primary agent driving morbidity and mortality during TSST-1-induced toxic shock in rabbits, as administration of Polymyxin B (an LPS inhibitor) or anti-endotoxin antibodies resulted in improved clinical status and survival [37, 42]. Furthermore, endotoxin-activated Kupffer cells secrete pro-inflammatory mediators contributing to toxicity and organ damage during TSS [26, 39]. Hence, in addition to the systemic effects of endotoxin, the high levels of endotoxin in the portal blood contribute to the direct injury to liver cells perpetuating the disease.

Importantly, an additional source of LPS has been recognized in women with menstrual TSS—vaginally present *E. coli*. Specifically, women who suffered menstrual TSS had *E. coli* co-colonizing their vaginas along with TSST-1-producing *S. aureus* [14]. If LPS produced by vaginal *E. coli* directly enters the systemic circulation, it may contribute to the development of TSS. In addition, *E. coli* was found to enhance the growth of TSST-1 producing *S. aureus* and its TSST-1 secretion [43]. This suggests that women vaginally colonized with both *E. coli* and *S. aureus* are at particularly high risk of developing severe TSS, as the disease is mediated by both, LPS entering the systemic circulation and secretion of high levels of TSST-1 [44].

The clinical systemic effects of TSST-1- and LPS-mediated shock, while not completely defined, are in part a result of the synergistic activation of several cellular and molecular pathways. TSST-1 induces an upregulation of an endotoxin receptor TLR4 on macrophages [45]. Portal blood endotoxin, via TLR4, activates Kupffer cells, which in turn produce the pro-inflammatory cytokine TNF-α and an array of reactive oxygen species (ROS). Both TNF-α and ROS induce liver tissue damage, thus perpetuating the impairment of liver's detoxification capabilities, and TNF-α is directly associated with TSS symptoms, such as fever [38, 46, 47]. TSST-1 also enhances LPS-induced secretion of other inflammatory cytokines and chemokines that contribute to the pathogenesis of TSS. Specifically, TSST-1 enhances LPS-mediated secretion of IFN-γ, interleukin-1β (IL-1β), IL-6, and IL-10 by human peripheral blood monocytes and spleens of rabbits [27, 48], IL-1 secretion in macrophages [49, 50], and TNF and IL-12 secretion by dendritic cells [45]. In addition, IFN-γ, a cytokine produced by TSST-1-activated T cells, was found to both mediate the sensitivity to endotoxin and enhance a liver damage, thus likely contributing to decreased LPS clearance [38, 51]. TSST-1 and LPS also synergize in their ability to induce IL-1 secretion from macrophages [49, 50]. Finally, TSST-1 was found to enhance LPS-induced renal tubular cell injury [52]. This synergistic and complex interaction between TSST-1 and LPS, induced inflammatory and toxic mediators, and multi-organ involvement warrants further investigation. Our goal is to define the cellular and molecular events that lead to endotoxemia during staphylococcal TSS and investigate the mechanisms of SAg and LPS synergism in the etiology of TSS utilizing rabbit models of infective endocarditis, sepsis, and pneumonia. Defining the cellular and molecular pathways underlying TSS will be instrumental for identifying effective therapeutic and preventive strategies to decrease TSS risk and burden during staphylococcal infections and increase survival.

Superantigen-mediated immune suppression

As mentioned above, SAgs are associated with many life-threatening *S. aureus* illnesses such as TSS, pneumonia, septicemia, and infective endocarditis. SAgs function by cross-linking the Vβ chain of the T-cell receptor (TCR) to the MHC II molecule on antigen-presenting cells independent of cognate antigen specificity, inducing a powerful activation of both T cells and macrophages with massive production of cytokines [53–57]. Normal T-cell responses usually lead to the activation of approximately 0.0001–0.001 % of the body's T-cell population, whereas SAgs can activate 20–30 % of T cells and in some cases up to 70 % of a person's total T-cell population [54]. Although immense, the SAg-mediated T-cell response is not *S. aureus*- or SAg-specific, as the SAg directs the response. The inflated number of activated T cells and macrophages secreting large amounts of cytokines is responsible for the clinical symptoms associated with SAgs and TSS, such as capillary leak, hypotension, rash, and fever [7].

Despite the massive SAg-mediated immune activation, clinical evidence suggests that the outcome of superantigenicity is actually immune suppression. It has been

shown that TSS, caused by TSST-1, reactivates endogenous viruses such as herpes simplex virus, previously misleading scientists to mistakenly conclude that a virus was the causative agent of TSS [58]. Histological evaluation of infections with group A streptococcus, a SAg-producing organism similar to *S. aureus*, revealed that few or no inflammatory cells were present in biopsies of deep tissue infections despite the presence of numerous streptococci organisms, extensive edema, and necrosis at the site of infection [59]. Inflammatory cells were found only upon exploration of deeper subdermal tissue, likely due to SAg-mediated immunosuppression although factors such as anti-inflammatory drug use by patients could have also played a role [59]. Other experiments showed that, like LPS, treatment with SEB could adequately stimulate monocytes to release superoxide but this treatment led to a significant decrease in superoxide release (hyporesponsiveness) by monocytes upon subsequent LPS or SEB exposure [60].

Defense against SAg-associated diseases relies on the host's ability to neutralize SAgs. Most individuals are exposed to SAgs early in life and develop antibodies to these proteins by age 20 with levels of TSST-1 antibody, for example, plateauing by age 40 [61]. For unknown reasons, 20 % of people in the USA never develop antibodies to any given SAg. This population is especially susceptible to SAg-associated diseases such as those caused by *S. aureus* and remains susceptible to SAgs even after repeated exposure. Individuals lacking SAg antibodies fail to develop specific immunity to any *S. aureus* components even after disease recovery. There is currently no definitive explanation for this lack of antibody generation in 20 % of the population and the reason(s) are likely to be multifactorial. One study showed that TSST-1 suppresses stimulated B-cell differentiation into immunoglobulin-secreting cells and that this suppression was not due to direct cytotoxic effects of SAgs on B and T cells, but rather a soluble factor (i.e. cytokines) produced by TSST-1-activated cells [62].

Experiments done in rabbits suggest that superantigenicity (massive activation of macrophages and $CD4^+$ T cells and the resultant cytokine storm) intoxicates adaptive immune cells, interfering with their ability to mount *S. aureus*-specific responses [63]. In rabbits immunized with TSST-1, only half develop detectable TSST-1 antibody titers, similar to humans where approximately 20 % do not make antibodies to SAgs. However, if SAgs are made inactive by mutating the host-cell binding sites that interact either with the Vβ T-cell receptor or the HLA class II molecule, 100 % of rabbits mount strong antibody responses not only to the SAg toxoid but also to other *S. aureus* virulence factors given concomitantly [63]. Hence, while wild-type SAgs suppress protective adaptive immune responses, as toxoids, they serve as adjuvants. It has been proposed that those individuals that do not generate antibodies to SAgs are incapable due to unknown genetic factors. However, data from the above study provide evidence that the host is in fact capable of mounting the correct immune response to the SAg (as a protein), but that the SAg action impedes this response.

Another study implicated antibody isotype as an important factor dictating an effective humoral response to SAgs. In this study, healthy subjects possessed TSST-1 antibodies that belonged exclusively to the IgG and IgM classes with IgG1 having most TSST-1 neutralizing capacity [64]. Most patients with menstrual TSS did not possess detectable levels of IgG1 anti-TSST-1 but rather, elevated levels of IgM, which were inadequate to neutralize TSST-1 [64]. This study also determined that several individuals were vaginally colonized with TSST-1-producing *S. aureus* and had low titers of neutralizing antibodies (IgG1); however, these individuals did not develop menstrual TSS. Therefore, a lack of protective anti-TSST-1 antibodies may be an important factor in menstrual TSS development, but additional factors are likely to be involved [64].

Taken together, these observations provide evidence that SAgs interfere with the development of protective immune responses. T-cell exhaustion is the most common explanation used to account for T-cell irresponsiveness during TSS. However, T-cell exhaustion has not been observed in humans with TSS. B-cell function, including activation state (lack of T-cell help), anergy, or chemotaxis/migration could be affected during TSS which would therefore inhibit the development of SAg- and *S. aureus*-specific antibodies, and memory responses. The mechanism by which SAgs inhibit antibody-mediated responses is poorly understood and the exact roles played by both T cells and B cells during SAg-mediated immunosuppression are still largely unknown and are the subjects of our current and future studies.

Conflict of interest None of the authors have conflicts of interest to declare.

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