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Staphylococcal superantigens: do they play a role in sepsis?

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Summary

In *Staphylococcus aureus*, 19 different superantigens (SAGs) have been described. Their genes are all located on mobile genetic elements, such as pathogenicity islands, plasmids, and phages. SAGs bypass conventional antigen recognition by directly cross-linking major histocompatibility complex class II (MHCII) molecules on antigen-presenting cells with T cell receptors. This leads to massive T cell proliferation and cytokine release, which may end in toxic shock syndrome. The role of SAGs in other forms of sepsis is less well defined. In animal models, SAGs and lipopolysaccharide (LPS) very efficiently synergize in the induction of lethal shock, and on the basis of these observations a two-hit model of sepsis has been proposed: LPS or another monocyte stimulus hits first, then SAG or another T cell stimulus hits. In clinical studies, however, evidence for an involvement of SAGs in sepsis has been difficult to obtain. This may have a number of reasons: differences between humans and rodents in their response to LPS and SAG, heterogeneity of SAG combinations in *S. aureus* clinical isolates, lack of tools to analyze SAG effects in patients, blocking anti-SAG serum antibodies, and MHCII polymorphisms.

Key words: superantigen • two-hit model • sepsis • *Staphylococcus aureus* • LPS • T cells

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INTRODUCTION

Staphylococcus (S.) aureus is a multifaceted bacterium which lives in a state of armed neutrality vis-a-vis mankind. It persists as a commensal bacterium in 10–30% of the human population, but it is also a common cause of food poisoning¹⁴². Beyond this, *S. aureus* can cause infections of varying severity, ranging from skin abscesses and wound infections to debilitating and even life-threatening diseases such as osteomyelitis, endocarditis, necrotizing pneumonia, toxic shock syndrome (TSS), and sepsis^{6, 86}. The pathogenicity of *S. aureus* is multifactorial. Its ability to cause such a broad range of diseases is due to an abundance of virulence factors which facilitate attachment, colonization, tissue invasion, toxinosis, and immune evasion⁶. However, host factors, environmental factors (e.g. intravascular catheters), and bacterial competition also contribute to the pathogenesis of staphylococcal infections⁸⁶. Furthermore, several studies have demonstrated that colonization with *S. aureus* is a significant risk factor for *S. aureus* infections^{25, 72, 143, 145, 148}.

Among the virulence factors of *S. aureus* are the superantigens (SAGs). SAGs are microbial exotoxins which activate large subpopulations of T lymphocytes, causing a massive cytokine release which may end in shock. In animal models it has been clearly demonstrated that SAGs can contribute to the pathogenesis of sepsis and septic shock. However, in clinical studies it has been difficult to show direct evidence for an involvement of SAGs, except for the rare cases of TSS.

The aims of this review are:

- to give an overview of the SAG spectrum encountered in *S. aureus*,
- to summarize recent data about the localization of the SAG genes on the *S. aureus* genome,
- to outline the evidence for a role of SAGs in sepsis,
- to discuss why it is so difficult to measure SAG effects in clinical situations.

S. AUREUS SUPERANTIGENS

SAGs are microbial toxins which activate large subpopulations of T lymphocytes by bypassing the physiological antigen processing and presentation pathways. Some of them are effective at femtomolar concentrations and belong to the most potent T cell mitogens known, so that the term “superantigen” appears very appropriate¹¹². SAGs can be secreted as exotoxins by different strains of *S. aureus*, *Streptococcus pyogenes*, *Streptococcus equi*, *Streptococcus dysgalactiae*, *Mycoplasma arthritidis*, and *Yersenia*

pseudotuberculosis, but there are also membrane-bound forms which are encoded in the genome of mouse mammary tumor viruses^{45, 112}. Thus, superantigenic toxins, which have a common mechanism of T cell activation, have evolved in parallel in very distant microorganisms.

This review focuses on the SAGs of *S. aureus*, where 19 different SAGs have been described: the TSS toxin (TSST)-1 and the staphylococcal enterotoxins (SE) A–R and SEU (Table 1). Due to the pace of detection of new SAGs, their nomenclature is still subject to change, which may give rise to confusion. In this article, we follow the recommendations of the International Nomenclature Committee for Staphylococcal Superantigen Nomenclature⁸³. The SAGs of *S. aureus* and *Streptococcus pyogenes* form the subgroup of pyrogenic toxin SAGs. Beside acting as SAGs, the members of this group are also pyrogenic and enhance an endotoxin shock in experimental models¹⁴. In addition, after ingestion the SE can cause staphylococcal food poisoning, a very acute gastroenteritis⁵⁹.

Superantigenicity

Conventional antigens are taken up and processed by antigen-presenting cells (APCs). The resulting antigenic peptides are bound to major histocompatibility complex (MHC) molecules and then displayed to T cells on the APC surface. These MHC/peptide complexes are recognized by T cells via the hypervariable loops of their T cell receptor (TCR) α and β chains. SAGs can bypass this highly specific interaction between T cells and MHCII/peptide complexes by directly cross-linking conserved structures on TCRV β chains with those on MHCII molecules. Both TCR and MHCII are contacted outside their antigen binding sites⁴⁵ (Fig. 1). Therefore, while SAG action strictly depends on the presence of MHCII molecules, it is, in contrast to conventional antigen presentation, not restricted by certain MHCII alleles. In spite of this, MHCII alleles may differ greatly in their efficiency of SAG-binding^{54, 61}.

On the T cell side, SAG binding is determined by the V β element used in the TCR. There are 47 functional V β elements in humans, which have been grouped into 23 TCRV β families on the basis of sequence similarities⁵⁶. Each SAG can bind to a subset of these V β elements, known as its V β signature. Usually, one to three TCRV β families dominate the response, while the involvement of others is less pronounced. For example, TSST-1 binds to all TCRV β 2-positive T cells, but in some cases activation of TCRV β 8.1-expressing T cells is also found (Table 1).

Table 1. Biochemical and functional properties of staphylococcal superantigens (SAGs)

SAG	Reference strain	Gen bank, accession number	Localization	MW (kDa)	Zinc binding	MHCII α/β chain	Emesis	Human TCR V β specificity	Reference
TSST-1	N315	NCBI, SA1819	SaPI1	21.9	–	+/-	no	2.1, 8.1	76
SEA	Mu50	NCBI, SAV1948	lysogenic phage (ϕ Sa3)	27.1	C-term	+/+	+	1.1, 5.3, 6.3 , 6.4, 6.9, 7.3-4 , 9.1, 16, 21.3, 22, 23.1	11
SEB	Col	TIGR, SA0907	SaPI1	28.4	–	+/-	+	1.1, 3.2 , 6.4, 12, 14, 15, 17, 20	151
SEC1	–	NCBI, X05815	SaPI1	27.5	cleft	+/-	+	3 , 3.2, 6.4, 6.9, 12, 15.1	
SEC2	–	NCBI, AY450554	SaPI1	27.6	cleft	+/-	+	12, 13.1, 13.2, 14, 15, 17, 20	
SEC3	N315	NCBI, SA1817	SaPI1	27.6	cleft	+/-	+	5.1, 12	76
SED	pIB485	NCBI, AF053140	plasmid (pIB485)	26.9	C-term, cleft	+/+	+	1.1, 5.3 , 6.9, 7.4, 8.1, 12.1	15, 154
SEE	FR1918	NCBI, M21319	bacteriophage?	26.8	C-term	+/+	+	5.1, 6.3-4, 6.9, 8.1	29
SEG	N315	NCBI, SA1642	SaPI3	27.0	–	+/-	+	3, 12, 13.1, 13.2, 13.6, 14 , 15	63, 76
SEH	MW2	NCBI, MW0051	SCCmec	25.2	C-term	-/+	nd	Vα10	11, 111
SEI	N315	NCBI, SA1646	SaPI3	24.9	nd	nd/+	+	1.1, 5.1 , 5.2, 5.3 , 6b , 23.1	63, 76
SEJ	pIB485	NCBI, AF053140	plasmid (pIB485, pF5)	28.5	nd	nd/+	nd	5.1 , 5.2, 6.7	15, 103, 154
SEK	Col	TIGR, SA0886	SaPI1	25.3	C-term?	nd/+	nd	5.1 , 5.2, 6.7	108, 151
SEL	N315	NCBI, SA1816	SaPI1	24.7	nd	nd/+	no	5.1 , 5.2, 6.7, 7, 9, 16, 22	76, 107
SEM	N315	NCBI, SA1647	SaPI3	24.8	nd	nd/+	nd	6a , 6b , 8, 9, 18, 21.3	63, 76
SEN	N315	NCBI, SA1643	SaPI3	26.1	nd	nd	nd	9	63, 76
SEO	N315	NCBI, SA1648	SaPI3	26.7	nd	nd	nd	5.1, 7, 9	63, 76
SEP	N315	NCBI, SA1761	lysogenic phage (ϕ Sa3)	26.4	nd	nd	nd	nd	11
SEQ	Col	TIGR, SA0887	SaPI1	26.0	nd	nd	no	2.1, 5.1 , 6.7 , 21.3	109, 151
SER	Fukuoka 5	NCBI, AB075606	plasmid (pIB485, pF5)	27.0	nd	nd	nd	3, 11, 12, 13.2, 14	103, 104
SEU	MRSA252	NCBI, SAR1918	SaPI3	nd	nd	nd	nd	nd	

Staphylococcal enterotoxins (SEs) and TSST-1 are localized on mobile genetic elements and they activate T cells in a TCRV β -specific manner (V β signature). Usually, one to three TCRV β families dominate the response (bold), while the involvement of others is less pronounced. Abbreviations: C-term – C-terminal, SE – staphylococcal enterotoxins, TSST-1 – toxic shock syndrome toxin-1, SaPI – staphylococcal pathogenicity island, SCCmec – staphylococcal chromosomal cassette encoding the methicillin resistance gene, MW – molecular weight, MHCII – major histocompatibility complex class II, TCR – T cell receptor, nd – not determined. For reference see also^{2, 27, 59, 110, 112}.

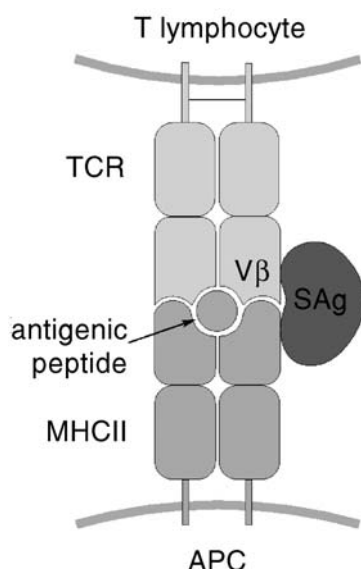


Figure 1. Superantigen function. Superantigens (SAGs) bypass the conventional antigen recognition by directly cross-linking major histocompatibility complex class II (MHCII) molecules on the antigen-presenting cell (APC) with T cell receptors (TCRs) on T lymphocytes.

Consequently, individual SAGs can activate large fractions (5–20%) of the T cell population. In contrast, conventionally processed peptide antigens are recognized by only 1 out of 10^4 – 10^5 naïve T cells. This V β -restricted T cell expansion is a characterizing feature of all SAGs^{28, 114}. Interestingly, there is one exception: the SAG SEH contacts TCRV α chains¹¹¹.

SAG structure and function

Pyrogenic toxin SAGs are a diverse group of proteins. SEB and SEK, for example, have only 15.5% amino acid sequence homology¹¹². However, resolution of their crystal structures has revealed a common three-dimensional structure, consisting of two globular domains, a C-terminal β -grasp motif (A domain), and a smaller N-terminal β -barrel domain (B domain)⁹³. The residues determining TCRV β specificity are located within the C-terminal region of the A domain, which is composed of four β -strands and a flanking α -helix. The B domain has an O/B (oligosaccharide/oligonucleotide-binding) fold, a common feature of different bacterial toxins. Interestingly, different mechanisms of MHCII binding have evolved within the family of pyrogenic toxin SAGs. Some SAGs have a low-affinity MHCII binding site in their B domain, which binds to the invariant $\alpha 1$ -domain of MHCII⁴⁵. Others have a second, zinc-dependent binding site in their A domain, which contacts the MHCII β -chain, so that they can cross-link MHCII molecules. Some SAGs can form dimers via

their zinc-binding sites, and these dimers then contact two MHCII molecules (see reviews^{12, 35, 114}; Table 1).

Effects on target cells

The cross-linking of MHCII and TCR by SAGs induces activation of both APCs and T lymphocytes. TCRV β -positive T cells proliferate and release large amounts of proinflammatory cytokines (IL-2, IFN- γ , and TNF- α). The T cell proliferation phase is followed by a profound state of unresponsiveness or even cell death. Therefore, an expansion as well as a reduction of the proportion of TCRV β -expressing T cells can be observed following SAG action^{28, 91}.

Monocyte activation requires dimerization of surface MHCII molecules and/or signaling via CD40⁸⁹. Both can be achieved by SAGs, since they bridge the membranes of APCs and T cells and further induce the expression of CD40-ligand by the T cells. In addition, SAGs with two MHCII-binding sites can induce MHCII cross-linking and thus activate monocytes independently of T cells^{41, 48, 81, 137}. Activated monocytes secrete TNF- α , IL-1 β , and IL-6 in response to the SAG stimulus.

The systemic release of proinflammatory cytokines by T cells and monocytes can be detected *in vivo* a few hours after a SAG stimulus. In severe cases this leads to generalized capillary leakage and hypotension. TNF- α and IFN- γ are considered to be the most important mediators of this SAG-induced shock. This TSS-like syndrome is commonly observed after injection of TSST-1 or enterotoxins into rodents⁹².

THE LOCALIZATION OF SAG GENES IN THE *S. AUREUS* GENOME

While the pathological effects of SAGs have been studied in detail, their physiological functions in bacterial life have remained elusive. Genetic analysis of *S. aureus* clinical isolates, including whole genome sequencing, has shown the following:

- 70–80% of all *S. aureus* clinical isolates harbor SAG genes, 5 on average^{58, 63, 117},
- the heterogeneity of the SAG repertoire between *S. aureus* strains is extensive^{16, 58, 63, 117},
- all staphylococcal SAG genes are localized on mobile genetic elements (Table 1).

Whole genome sequencing has revealed that staphylococcal SAG genes are encoded by accessory genetic elements that are either mobile or were formerly mobile, i.e. plasmids, prophages, transposons, and pathogenicity islands (Table 1)^{11, 57, 76}. The presence of SAG genes on mobile elements along with other virulence factors probably facilitates their horizontal

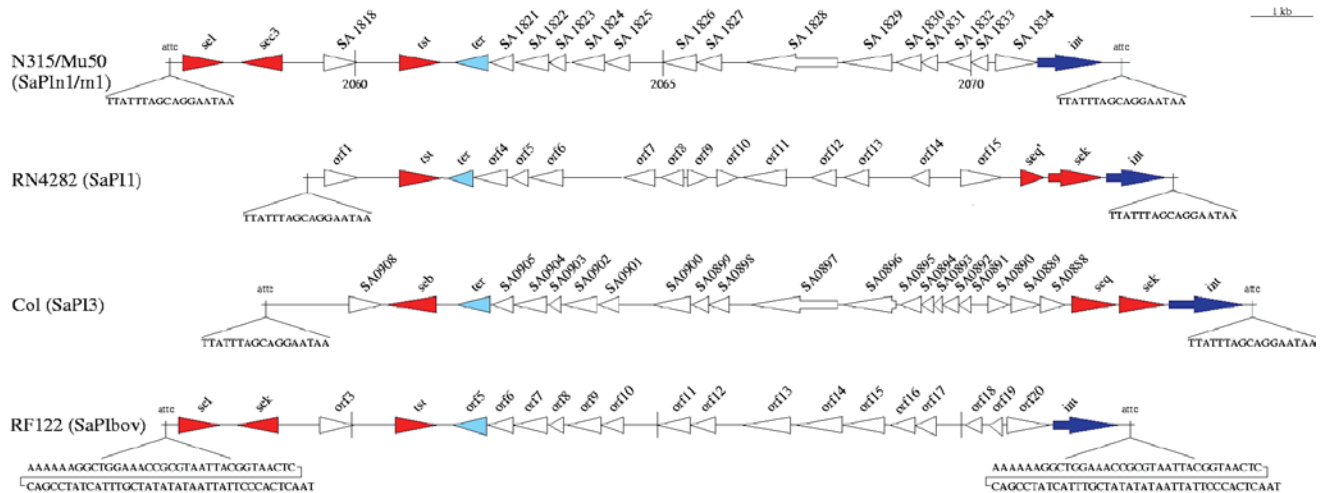
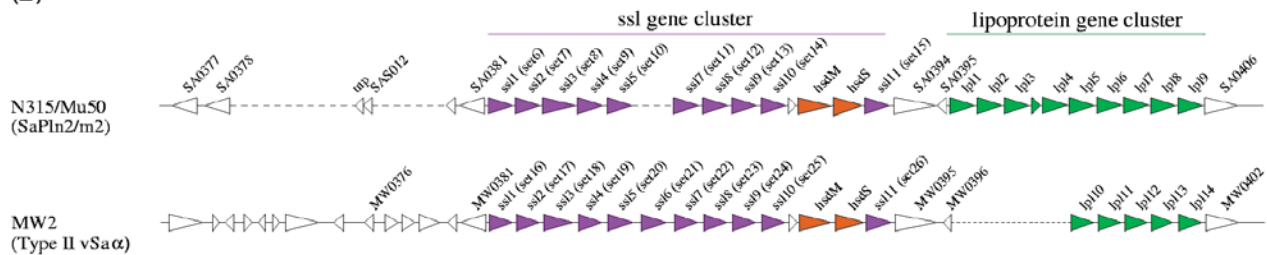
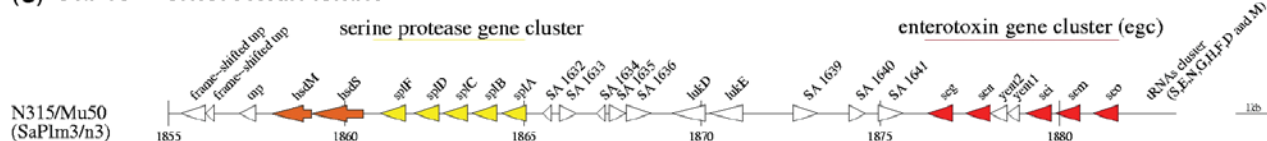
(A) SaPI1 – TSST-1 islands**(B) SaPI2 – SSL islands****(C) SaPI3 – enterotoxin island**

Figure 2. Staphylococcal pathogenicity islands. The staphylococcal pathogenicity islands 1-3 (SaPI1-3) carry superantigen (SAG) genes or staphylococcal superantigen-like (ssl) genes^{11, 76, 151}. The PAI nomenclature is adapted from Kuroda et al.⁷⁶, synonyms are shown in brackets. **A** – SaPI1, **B** – SaPI2, **C** – SaPI3. Arrows and arrowheads indicate open reading frames (ORF) and their direction of transcription, while broken lines symbolize missing ORFs. Abbreviations: tnp – transposase gene, int – integrase gene, luk – leucotoxin gene. Color scheme: red – superantigens, magenta – ssl, yellow – serine proteases, green – lipoproteins, blue – integrases, light blue – likely terminase genes, brown – restriction/modification system, white – ORFs with unknown function.

spread between *S. aureus* strains^{43, 57}. In fact, a comparison of the 5 published *S. aureus* genomes (N315, Mu50, MW2, MRSA252 and MSSA476^{11, 57, 76}) showed marked variation in the distribution and composition of these mobile elements (Fig. 2). This is reflected by the extensive heterogeneity of the SAG repertoire in *S. aureus* isolates.

Pathogenicity islands

The pathogenicity islands (PAI) of *S. aureus* are the first clearly defined PAIs in Gram-positive bacteria.

PAIs have evolved from former lysogenic bacteriophages and plasmids, and they are defined as large genomic regions (>15 kb) which are commonly present in pathogenic variants, but not in closely related non-pathogenic bacteria. PAIs carry virulence-associated genes, differ in their G+C content from the rest of the chromosomal genome, are flanked by direct repeats, and carry mobility genes, including conserved integrases^{49, 151}. They are widely assumed to be mobile; however, of the staphylococcal PAIs, mobility has only been demonstrated for SaPI1bov⁸⁴. Recently, variants of the staphylococcal PAIs have been discovered

which lack virulence genes, so that the more general term “genomic island” is sometimes preferred^{11, 38}.

SaPI1 (TSST-1 island)

The *S. aureus* pathogenicity island (SaPI)1 is the prototypic staphylococcal PAI^{84, 118}. It is 15.2 kb in length and is flanked by 17-bp direct repeats (*att*_c). SaPI1 encodes the SAgS TSST-1, SEK (formerly entK), and SEQ (formerly SEI), and it carries a functional integrase gene¹¹⁸. Lindsay et al.⁸⁴ have demonstrated that SaPI1 can be mobilized by the helper phage Φ80α. During the vegetative growth of Φ80α, the genomic island is excised from its unique chromosomal insertion site *att*_c, amplified, and encapsidated into specialized phage heads. After transduction to a *recA*-deficient *S. aureus* recipient strain, SaPI1 integrates at the *att*_c site, presumably directed by the self-encoded integrase¹¹⁸. In the absence of a helper phage the island is very stable.

Several variants of SaPI1 have been described, which differ in their SAg genes (Fig. 2A). SaPIbov from a bovine mastitis isolate contains *tst*, a *sec* variant, and *sel*. The SaPI1 homologue of the *S. aureus* reference strain COL, which has been named SaPI3 (not to be confused with the SaPI3 described below), contains *seb* at the same position as *tst* in SaPI1 and, additionally, *sek* and *seq*^{108, 109}. This explains the phenomenon of toxin gene exclusion: *seb* (on SaPI3) and *tst* (on SaPI1) never coexist in a clinical *S. aureus* isolate¹⁵¹.

SaPI3 (enterotoxin island)

The SaPI3 is composed of a serin protease gene cluster, a leucocidin gene cluster (*lukD*, *lukE*), and an enterotoxin gene cluster (*egc*) (Fig. 2C)⁷⁶. The *egc* contains five SAg genes, *seg*, *sei*, *sem*, *sen*, and *seo*, as well as two pseudogenes with sequence homology to enterotoxin genes, ψ *ent1* and ψ *ent2*^{63, 76}. Recently, a new putative enterotoxin gene locus *seu* has been discovered which results from a gain of function mutation in the pseudogenes⁷⁹. Based on phylogenetic analyses, Lina et al.⁸³ have suggested that the *egc* may be the enterotoxin nursery from which all known *S. aureus* SAg genes have evolved. *egc* SAgS are the most frequent SAgS in *S. aureus*, as 50–60 % of clinical isolates contain this gene cluster, but so far they could not be clearly associated with clinical syndromes^{58, 62, 63, 88, 90, 105, 117}. Transcriptional analysis revealed that the *egc* functions as an operon⁶³.

Prophages

All sequenced *S. aureus* strains (except for COL) have the prophage ΦSa3 integrated into their chromosomes^{11, 57}. There are several variants of this bacterio-

phage: The *S. aureus* strain Mu50, for example, carries an *sea* gene on ΦSa3, while in N315 *sep* is present at the same locus^{11, 76}. MW2 and the closely related strain MSSA476 harbor *sea*, and, additionally, genes for *seg2* and *sek2*, the latter two being homologues to *seg* and *sek*, respectively, which are found on SaPI1 in COL^{11, 57}. ΦSa3 is generally mobile, but some defective variants have been described^{17, 129}. The gene for SEE, *see*, is probably also located on a phage²⁹.

Plasmids

The genes for SED, SEJ, and the recently discovered SER are carried by plasmids. *sed* and *sej* are colocalized on the penicillinase plasmid pIB485^{15, 154}. The *ser* gene is either located together with *sed* and *sej* on a pIB485-related plasmid, or only with *sej* on a pF5-related plasmid^{103, 104}.

SaPI2 (SSL island)

On SaPI2 a large family of putative exotoxins has been identified which share sequence homology with superantigens^{11, 76}. These proteins were discovered by Williams et al.¹⁴⁹, who described a cluster of five related genes in *S. aureus*, which they called SE-like genes, *set1*–*set5*. Comparison of *set*-clusters from different strains showed a remarkable heterogeneity in both gene number (7–11 genes) and the encoded peptide sequences⁴⁴. Recently, the International Nomenclature Committee for Staphylococcal Superantigen Nomenclature recommended that the SET family should be renamed staphylococcal superantigen-like (SSL) proteins and that the encoding genes should be designated *ssl1*–*ssl11*⁸³ (Fig. 2B). Their function is still unknown. Despite their sequence homology to SAgS, SSL-proteins do not have SAg properties^{7, 44}. Crystal structure analysis revealed a structural similarity with SAgS; however, the residues which are important for MHCII and TCR binding are not conserved^{3, 7}. Recombinant SSL1 has been shown to induce IL-1β, IL-6, and TNF in peripheral blood mononuclear cells¹⁴⁹. Moreover, SSL5 and SSL7 have been demonstrated to interact with monocytes and dendritic cells³. We propose that SSL proteins play a role in the interaction of *S. aureus* with its host because, first, the *ssl* gene cluster is located on a pathogenicity island and, second, antibodies against SSL proteins are highly prevalent, implying that the SSL proteins are secreted in the host^{11, 44, 76}. The fact that the cluster is present in every *S. aureus* strain indicates that it is essential⁵⁸.

DIFFERENT PATHWAYS INTO SEPTIC SHOCK

Sepsis is defined as a systemic inflammatory response to an infection. The causative agents are microorgan-

isms or their toxins, which spread from a local infection site and enter the blood stream. Severe sepsis is complicated by organ dysfunction, and the term septic shock refers to the subsequent state of acute circulatory failure⁸⁰. Sepsis and septic shock are the major causes of death in intensive care units^{4, 39}. Recent USA and European epidemiological studies have reported that severe sepsis accounts for 2–11% of all hospital or intensive care unit admissions and that each year it causes the death of 200,000 patients in the USA alone⁵. While Gram-negative infections were the predominant cause in the 1960s and 1970s, the incidence of Gram-positive infections increased in the past two decades. Today, Gram-positive organisms account for about half of the cases of severe sepsis^{22, 130}. Despite intensive research and improvements in supportive care, hospital mortality of severe sepsis and septic shock has remained frighteningly high: 30 and 60%, respectively^{19, 46}.

Gram-negative sepsis

The pathophysiology of Gram-negative sepsis has been thoroughly studied in the last decades and it is now understood in great detail. Lipopolysaccharide (LPS), or endotoxin, which is a major component of the outer cell membrane of Gram-negative bacteria, plays a key role. LPS acts as a pathogen-associated molecular pat-

tern (PAMP) and it very strongly activates monocytes and macrophages, which recognize minute amounts of the endotoxin via specific pattern-recognition receptors. The elucidation of the very complex recognition process constitutes a milestone in immunological research. LPS is bound by the soluble LPS-binding protein (LBP) and transferred to a membrane-bound receptor complex on monocytes/macrophages, which is composed of CD14, Toll-like receptor (TLR)4, and myeloid differential protein-2 (MD-2)^{125, 127, 150} (Fig. 3). CD14, a GPI-anchored protein, probably helps to load LPS onto the TLR4-MD-2 complexes¹. The signal is then transduced by TLR4 and results in the activation of the transcription factor NF κ B. In response to this signal, monocytes and macrophages synthesize and secrete large amounts of proinflammatory mediators, such as TNF, IL-1, IL-6, chemokines, platelet activating factor, leukotriens, colony-stimulating factors, oxygen radicals, and nitric oxide (NO). These mediators contribute to sepsis pathogenesis by inducing vascular leakage, hypotension (especially via NO), hemoconcentration, and metabolic acidosis^{8, 130}.

Gram-positive sepsis

S. aureus, coagulase-negative staphylococci and streptococci are the most common causes of Gram-positive sepsis. In contrast to Gram-negative organ-

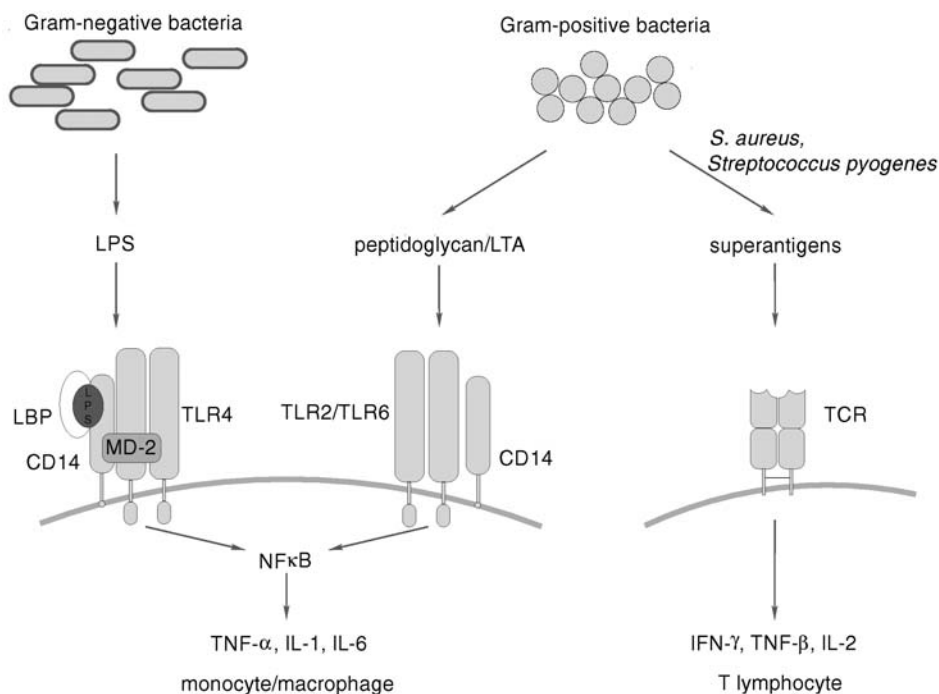


Figure 3. Different pathways into septic shock. Two pathways for triggering lethal shock can be defined: Cell wall components from Gram-negative and Gram-positive bacteria (LPS, peptidoglycan/LTA) interact with pattern-recognition receptors on monocytes, while SAGs activate T lymphocytes. Monocytes and T cells are triggered to release large amounts of proinflammatory cytokines which can eventually induce lethal shock. While TNF- α is the most important cytokine in monocyte-mediated shock, IFN- γ plays a key role in SAG-induced shock. Abbreviations: LPS – lipopolysaccharide, LBP – LPS binding protein, MD-2 – myeloid differential protein 2, TLR – Toll-like receptor, LTA – lipoteichoic acid, TCR – T cell receptor.

isms, these bacteria do not contain LPS. However, they produce a variety of extrinsic and intrinsic molecules which can trigger inflammation: cell wall components, such as peptidoglycans and lipoteichoic acid (LTA) on the one hand, and exotoxins, such as SAg, on the other²⁴.

Similarly to LPS, LTA and peptidoglycans as well as whole bacteria are recognized by monocytes and macrophages via the pattern-recognition receptors TLR2 and, probably, TLR6^{24, 55, 78, 146} (Fig. 3). This induces the release of proinflammatory cytokines (TNF- α , IL-1, IL-6, and IL-8) and NO. Additionally, peptidoglycan seems to activate coagulation and alternative complement pathways^{22, 70}. Moreover, LTA and peptidoglycan synergize in the induction of NO formation and the release of TNF and IFN- γ ⁷¹ and, importantly, they act synergistically in inducing shock and multiorgan failure in rats¹⁴¹. However, in comparison with LPS, their potency is low⁷⁸.

While LPS as well as cell wall components of Gram-positive bacteria predominantly activate monocytes, macrophages, and other cells of the innate immune system, SAg primarily target T lymphocytes. As described above, SAg induce a massive cytokine release from T cells and monocytes, which in rare cases can lead to TSS, which is characterized by fever, hypotension, rash, desquamation, and multiorgan failure^{87, 133}.

TSS – SAg-induced shock

As described above, TSS can be considered as a form of Gram-positive sepsis, which is dominated by the pathophysiological effects of staphylococcal or streptococcal SAg. TSS is a rare disease with an incidence of 1/100,000⁵⁰. It occurs most frequently in young women during their menses due to the usage of high-absorbency tampons, which facilitate the growth of *S. aureus* (menstrual TSS), but it can also be a complication of surgery, burns, and wound infections (non-menstrual TSS)^{50, 87}. The causative agent of menstrual TSS is TSST-1, and a single *S. aureus* clone has been shown to be responsible for the majority of menstrual TSS cases⁹⁴. The strict association of TSST-1 with menstrual TSS is probably due to its unique ability to cross mucosal barriers¹²². Non-menstrual TSS cases represent one third of all TSS cases today⁵⁰ and they are associated with TSST-1 or other staphylococcal SAg^{20, 21, 123}.

Several studies have unequivocally demonstrated SAg effects during clinical streptococcal or staphylococcal TSS. First, the selective activation and expansion (or deletion) of T cells corresponding to the

TCRV β signature of the suspected SAg has been reported in TSS patients^{28, 91, 147}; for example, up to 70% of the peripheral T cells were V β 2 positive in a case of TSST-1-induced TSS²⁸. Second, in some cases serum samples from TSS patients are mitogenic for T cells, which indicates the presence of SAg^{97, 113}. Additionally, in rare cases SAg could be directly measured in the serum of TSS patients^{113, 131}. Third, SAg-neutralizing antibodies are beneficial in streptococcal TSS, and treatment with intravenous immunoglobulins reduces mortality^{33, 69, 100, 101}.

THE TWO-HIT MODEL OF SEPTIC SHOCK

Animal models confirm that two pathways into septic shock can be distinguished, one dominated by the innate, the other by the adaptive immune system: On the one hand, SCID-mice, which lack B and T cells, are resistant to SEB-induced shock, but sensitive to LPS. This SAg resistance can be reversed by reconstitution with T cells⁹². On the other hand, SEB can cause lethal shock in endotoxin-resistant C3H/HeJ mice, which lack functional TLR4, despite their deficient macrophage response¹⁵². Since both pathways lead to the release of large amounts of proinflammatory cytokines and eventually to lethal shock, the question arose whether these pathways may synergize. In most immune responses the innate and the adaptive immune systems cooperate, and the multitude of synergistic and antagonistic interactions at the interfaces of their intricate network are in the focus of intensive research. It appears that an extreme dominance of either the innate or the adaptive response is probably the exception rather than the rule: In an organism which is colonized with huge numbers of different microorganisms, the isolated exposure to stimuli of the innate immune system, such as LPS (or LTA and peptidoglycans), or to stimuli of the adaptive immune system, such as SAg, may be a rare event outside of the laboratory. These considerations and the observation that LPS and SAg synergize in rodents have motivated the development of the two-hit model of septic shock by Bannan et al¹³. They suggested that in septic shock the following sequence of events may be typical: A Gram-negative infection causes symptoms of vasodilatation and hypotension (1st hit). Treatment with fluids and antibiotics rescues the patient. However, some days later a Gram-positive insult, typically originating from the skin or the gastrointestinal flora, causes an irreversible shock in the LPS-sensitized patient (2nd hit)¹³. While the sequence of events may differ from the original hypothesis, there is now a large body of experimental data which supports the two-hit model: 1st hit – LPS or another monocyte stimulus; 2nd hit – SAg or another T cell stimulus.

Synergism of SAg and LPS in shock induction

Co-injection of LPS and SAg (SEB) in mice reduces the lethal dose for both shock inducers almost 100-fold and enhances the release of TNF- α , IL-6, and IFN- γ ¹⁸. This synergism is T cell dependent and effectively prevented by cyclosporin A. The key mediator appears to be IFN- γ , since neutralizing antibodies to it are inhibitory¹⁸. The most impressive form of synergism is observed when animals are primed with a sublethal dose of SAg followed by an injection of endotoxin a few hours later^{18, 31, 37, 120, 134}. The group of Schlievert studied the kinetics of TSST-1-induced LPS enhancement in a rabbit model. Depending on the doses of TSST-1 and endotoxin given, rabbits showed an up to 50,000-fold enhanced susceptibility to either SAg or endotoxin¹²⁰. Similarly, the injection of a moderate dose of the SAgS SEB or TSST-1, which was not able to trigger a lethal cytokine syndrome, increased the sensitivity to endotoxin in mice^{31, 37}. A sublethal priming injection of TSST-1 12 hours before LPS-injection reduced the lethal dose of LPS 20-fold and induced a 1000-fold increase in serum TNF- α levels³⁷. T cells were essential for this SAg-mediated LPS sensitization, because T cell-deficient SCID mice neither upregulated TNF- α serum levels nor exhibited enhanced lethality^{31, 37}. Additionally, the SAg effect was reconstituted by adoptive T cell transfer³⁷. Cyclosporin A treatment and anti-IFN- γ antibodies were protective and strongly reduced TNF- α serum levels³⁷. Altogether, these data clearly show that the activation of T cells is the basis of the SAg-mediated LPS-priming in mice.

What are the sources of LPS in SAg-primed organisms? The commensal Gram-negative gut flora probably plays an important role. Low levels of circulating endotoxin can be detected even in healthy humans⁶⁴, and increased intestinal absorption as well as reduced endotoxin clearance can lead to elevated endotoxin plasma concentrations^{65, 82, 116, 136}. This has been observed in patients with liver cirrhosis, hemorrhagic shock, cardiac surgery, and severe acute pancreatitis^{51, 64, 115, 138}. An elevation of endogenous circulating LPS may even be caused by the SAg itself, since SAg-induced hypotension with splanchnic hypoperfusion and ischemia injury damages the intestinal barrier function, so that Gram-negative bacteria and/or endotoxin can translocate across the gut wall^{82, 115}. SAgS (in synergy with LPS) may also be toxic for hepatocytes^{95, 121}, which are crucially involved in endotoxin clearance^{65, 82}.

There is evidence that endogenous endotoxins also contribute to the severity of TSS: In rabbits TSST-1

treatment increased the levels of circulating endotoxin, and death could be prevented by co-administration of the LPS-neutralizing drug polymyxin B¹³⁶. Similarly, in a study with 10 human TSS patients the serum concentration of endotoxin was increased in the acute phase and returned to normal values in convalescence¹³⁶. These data show that the intestine can act as an endogenous source for endotoxin. Increases in LPS translocation, especially when combined with reduced endotoxin clearance, may lead to LPS serum concentrations which, in synergy with SAgS, may become life threatening.

Co-infecting Gram-negative bacteria could be an exogenous source of LPS in Gram-positive sepsis. Large proportions of patients with non-Gram-negative sepsis show endotoxemia, and TSS patients frequently acquire opportunistic infections with Gram-negative bacteria, such as *Haemophilus influenza*, *Pseudomonas aeruginosa*, and *Escherichia coli*^{32, 60, 120}. Polymicrobial infections are reported to account for approximately 10% of bacteremic episodes^{9, 128}. However, the incidence of polymicrobial infections may still be underestimated, because they are difficult to detect^{106, 130}.

The SAgS SEA, SEB and TSST-1 strongly enhance the LPS-induced production of cytokines, such as TNF- α , IFN- γ , and IL-6^{18, 53, 134}. TNF- α then plays a key role, because in SAg-primed mice, which lack the p55 TNF-receptor, LPS is not lethal¹⁸. The SAg-induced rise in TNF- α secretion is mainly mediated by the T cell-derived IFN- γ , a strong macrophage activator. Purified IFN- γ enhances TNF- α synthesis by LPS-stimulated monocytes *in vitro* and it induces LPS hypersensitivity in mice, whereas *in vivo* neutralization of IFN- γ activity prevents it^{18, 23, 37, 47, 52, 67}. Bosisio et al.²³ showed that IFN- γ increases the expression of the LPS receptors TLR4 and CD14 by monocytes, which could explain the sensitizing effect. Conversely, short-term preincubation with TLR ligands, e.g. LPS, amplifies the IFN- γ signaling via increased phosphorylation of STAT1^{30, 75}. In the effector phase, IFN- γ augments the toxic TNF- α effects on different tissues¹⁴⁰. In addition to IFN- γ , secretion of GM-CSF and surface expression of CD40-ligand by SAg-activated T cells may contribute to the overwhelming monocyte activation, which is the hallmark of LPS sensitization.

DO SAGS AND ENDOTOXIN SYNERGIZE IN HUMAN SEPSIS?

Data from animal models have convincingly demonstrated that SAgS and LPS very efficiently synergize in the induction of lethal shock. If SAgS could also

sensitize humans to LPS, this might have serious consequences given the high toxicity of endotoxin in man: The lethal LPS dose of 1–2 μg would be reduced to ng or even pg amounts, assuming a similar amplification¹¹⁹. However, evidence for the relevance of the two-hit model in human sepsis has been difficult to obtain. This may have a number of reasons:

1. differences between humans and rodents in their response to LPS and SAg,
2. heterogeneity of SAg combinations in *S. aureus* clinical isolates,
3. lack of tools to analyze the SAg effects in patients,
4. blocking anti-SAg serum antibodies, and
5. MHCII polymorphisms.

1. Differences between humans and rodents in their response to LPS and SAg

Interactions of SAg and endotoxin are usually assessed in mice and rabbits. In comparison with humans, their susceptibility to the toxicity of LPS and SAg is low, because small sequence differences in the cellular toxin receptors result in much lower affinities^{36, 42, 132, 153}. Therefore, in humans even minute concentrations of SAg and/or LPS, which are below the level of detection, might have strong effects.

*2. Heterogeneity of SAg combinations in *S. aureus* clinical isolates*

In mouse models of SAg-induced shock a single purified or recombinant SAg is usually injected into the animal. However, a recent survey revealed that clinical *S. aureus* isolates harbor five SAg genes on average⁵⁸. The effects of complex “SAg cocktails” on the pathogenesis of severe systemic infections is poorly understood.

3. Lack of tools to analyze the SAg effects in patients

By definition, SAg activate T cells in a TCRV β -restricted manner. Therefore, SAg effects in patients are usually measured by a shift in the TCRV β repertoire of the T cell pool. However, the characterization of the V β signatures of individual SAg is complicated by several factors. First, SAg effects may be variably associated with T cell proliferation or apoptosis. Second, the T cell responses are influenced by MHCII polymorphisms⁸⁵. Finally, while the V β signature may predict the shift in the TCRV β repertoire in *S. aureus* strains with only a single SAg gene, most *S. aureus* strains carry multiple SAg genes, and in these cases their composite V β signature cannot be inferred from the determined SAg gene repertoire (Holtfreter,

unpublished data). Therefore, the analysis of the T lymphocyte V β subset composition does not appear to be suitable for the detection of SAg involvement in most cases of sepsis and related illnesses.

Direct measurements of SAg in serum have only rarely been successful, since SAg on their own are effective in femtomolar concentrations, and synergism with LPS may occur at even lower concentrations. However, in a recent study, circulating staphylococcal and streptococcal SAg were detected in 5/16 sepsis patients and 10/24 patients with septic shock¹⁰. Additionally, the streptococcal SAg SPEA has been detected in the serum of 4/7 patients with streptococcal TSS or invasive disease immediately after admission¹³¹. Furthermore, it has been shown that anti-SAg antibody titers rise after staphylococcal infections, such as wound infections or septicemia^{66, 68}. However, while serum conversion indicates a systemic release of SAg during infection, it allows no conclusions about the SAg effects on T cells.

4. Blocking anti-SAg serum antibodies

SAg are strongly immunogenic and there is a high prevalence of antibodies against SEA, SEB, SEC, SED, and TSST-1 in the healthy community^{66, 77, 102, 124}. These antibodies can neutralize SAg and abolish their proliferative effects on T cells⁵⁸. A notable exception are the SAg encoded by the *egc* on SaPI3 (Fig. 2C), which are the most prevalent SAg in *S. aureus*⁶³. Surprisingly, neutralizing antibodies against the *egc*-encoded SAg are very rare⁵⁸. Seroconversion against SAg has been observed in patients with *S. aureus* septicemia, though minor infections and possibly even *S. aureus* carriage may also induce an anti-SAg antibody response^{66, 68}.

Evidence for a protective role of neutralizing anti-SAg antibodies is abundant: While more than 90% of healthy men and women older than 25 years have high anti-TSST-1 antibody titers, these are absent in 90% of patients with menstruation-associated TSS^{34, 87, 135, 144}. A lack of specific antibodies was also found in invasive streptococcal disease^{14, 40, 101}. Intravenous Ig preparations (IVIG), which are prepared from large pools of human plasma, contain considerable amounts of neutralizing anti-SAg antibodies, and treatment with IVIG reduced cytokine secretion, bacterial load, and mortality in streptococcal TSS^{33, 69, 96, 99, 100, 139}. Moreover, there is anecdotal evidence that an IVIG-therapy can also decrease mortality in staphylococcal TSS⁸⁷.

Therefore, when analyzing the role of SAg in a clinical context, the patient's antibody status has to be

taken into account, because it can skew or even abolish the SAg effects on T cells.

5. MHCII polymorphisms

The impact of host genetic factors on the susceptibility to infections as well as on outcome has come into the focus of research, and the influences of age, gender, cytokine genes and, most importantly, MHC genes have been discussed^{74, 126}. Host factors which influence the outcome of systemic infections with *Streptococcus pyogenes* have been studied in detail. A single clone of *Streptococcus pyogenes* can cause clinical syndromes of varying severity, ranging from superficial carriage through pharyngitis to toxic shock syndrome^{14, 26, 98}. Recently, MHCII haplotypes were identified to be a key factor in host susceptibility to severe infections with *Streptococcus pyogenes*: Certain MHCII haplotypes are protective, while others increase the risk of disease⁷³. Llewelyn et al.⁸⁵ have demonstrated that the SPEA binding affinity to different MHC-DQ alleles varies significantly, which results in dramatic differences in T cell proliferation and cytokine production, and which even influences the TCRV β repertoire of the stimulated T cells. Especially at low SAg concentrations, as might be encountered during sepsis, only strongly binding MHCII alleles mediate TNF- α secretion⁸⁵. However, the possibility that MHC polymorphisms could influence superantigenicity and thus clinical susceptibility to the toxicity of individual superantigens has received little attention until recently. While the findings do not contradict the well-established notion

that SAg action strictly depends on the presence of MHCII molecules but is not restricted by certain MHCII alleles (as is the presentation of conventional antigens), they add one more level of complexity to the analysis of SAg effects in patients.

CONCLUSIONS

In experimental settings, shock can be induced by LPS, which activates monocytes via pattern-recognition receptors, and also by SAgS, which activate large T cell subpopulations. The two pathways are interconnected and potentiate each other. We suggest that the two-hit model of sepsis should be generalized, because in most cases of systemic bacterial infections both the innate and the adaptive immune system will be stimulated simultaneously or sequentially. The 1st hit would comprise all PAMPs which can activate the cells of the innate immune system after binding to their pattern-recognition receptors. The 2nd hit would include SAgS and other T cell stimuli, for example recall antigens, which elicit a fast and strong memory response. TSS, with its strong bias on T cell stimulation, could be regarded as one extreme form of such a two-hit scenario, while the experimental endotoxin shock would represent the other end of the spectrum.

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