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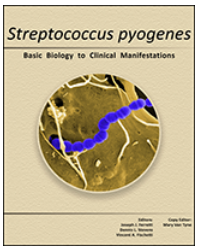

Streptococcal Superantigens: Biological properties and potential role in disease

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Abstract

Superantigens (SAgs) are a family of highly potent mitogens that share the ability to trigger excessive stimulation of human and other mammalian T lymphocytes. This leads to a massive release of T cell mediators and pro-inflammatory cytokines contributing to diseases such as toxic shock syndrome. In contrast to conventional peptides, SAgs bind as unprocessed molecules to major histocompatibility (MHC) class II molecules outside the peptide-binding groove and sequentially to the variable β-chain of the T cell receptor (TcRVβ). Currently, eleven *Streptococcus pyogenes* SAgs are described in the literature. Together with the SAgs produced by *Staphylococcus aureus*, they build a larger family of structurally related, heat-stable exotoxins.

This chapter provides a comprehensive overview of the discovery, biological function, and disease-associations of these remarkable proteins.

Introduction

Superantigens (SAgs) are a family of highly mitogenic exotoxins that are produced by a small number of bacterial species and some viruses (Fraser & Proft, 2008; Proft, Schrage, & Fraser, 2005) (Fraser & Proft, 2008; Proft, Schrage, & Fraser, 2005). The most common bacterial genus that produces SAgs is *Streptococcus spp*. and includes *Streptococcus pyogenes* (group A streptococcus), *S. dysgalactiae* (group C Streptococcus) and *S. equi* (group G streptococcus) (Proft, Schrage, & Fraser, 2005; Commons, Smeesters, Proft, Fraser, Robins-Browne, & Curtis, 2014; Proft & Fraser, 2003). A variety of SAgs are also found in *Staphylococcus aureus* and coagulase negative staphylococci, which together with the streptococcal SAgs, build a family of structurally related low molecular weight exotoxins, with secretion dependent on a cleavable signal peptide sequence (Fraser & Proft, 2008; Proft & Fraser, 2003; Baker & Acharya, 2004). Other, structurally non-related SAgs are produced by *Mycoplasma arthritidis* (Proft, Schrage, & Fraser, 2005; Rink & Kirchner, 1992) and *Yersinia pseudotuberculosis* (Proft, Schrage, & Fraser, 2005; Donadini & Fields, 2007).

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A hallmark of SAgs is their ability to simultaneously bind to major histocompatibility complex (MHC) class II on antigen presenting cells and the T cell receptor on T cells (Fraser & Proft, 2008; Proft & Fraser, 2003; Proft & Fraser, 2007). In contrast to conventional peptide antigens, SAg binding is not restricted by polymorphic determinants of MHC class II molecules and occurs outside the peptide-binding groove. Furthermore, SAgs bind to the variable region of the TcRVβ chain, resulting in extensive heterogeneity in T cell clonal activation (Figure 1). The number of different TcR β-chains in the human T cell repertoire is restricted to less than 50 with only about 25 major Vβ types. Since SAgs generally bind more than one specific Vβ region, up to 25% of an individual's T cell population can be activated, which is in sharp contrast to 1 in 10^5-10^6 naïve T cells that are stimulated in response to conventional peptide antigens. Consequently, each SAg is associated with a characteristic TcRVβ 'fingerprint' that is independent from MHC class II polymorphism (Table 1). For example, streptococcal pyrogenic exotoxin (SPE)-C triggers the activation and expansion of T cells carrying Vβ2.1, Vβ3.2, Vβ12.5 and Vβ15.1 with a strong preference for Vβ2.1, whereas streptococcal mitogenic exotoxin (SMEZ) shows specificity for Vβ2.1, Vβ4.1, Vβ7.3 and Vβ8.1 regions with a preference for Vβ4.1 and Vβ8.1 (Table 1). Due to the immense potency to stimulate human, and to a certain degree, other mammalian CD4 and CD8 T cells, the term 'superantigen' was introduced by Philippa Marrack and John Kappler in 1989 (White, et al., 1989). In response to the oligoclonal activation of T cells and antigen presenting cells massive amounts of proinflammatory cytokines, such as interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and T cell mediators, such as IL-2 are released. This 'cytokine storm' can lead to fever and shock. With half-maximum responses between 0.02 and 50 pg/ml for human T cells, SAgs are the most potent T cell mitogens ever discovered.

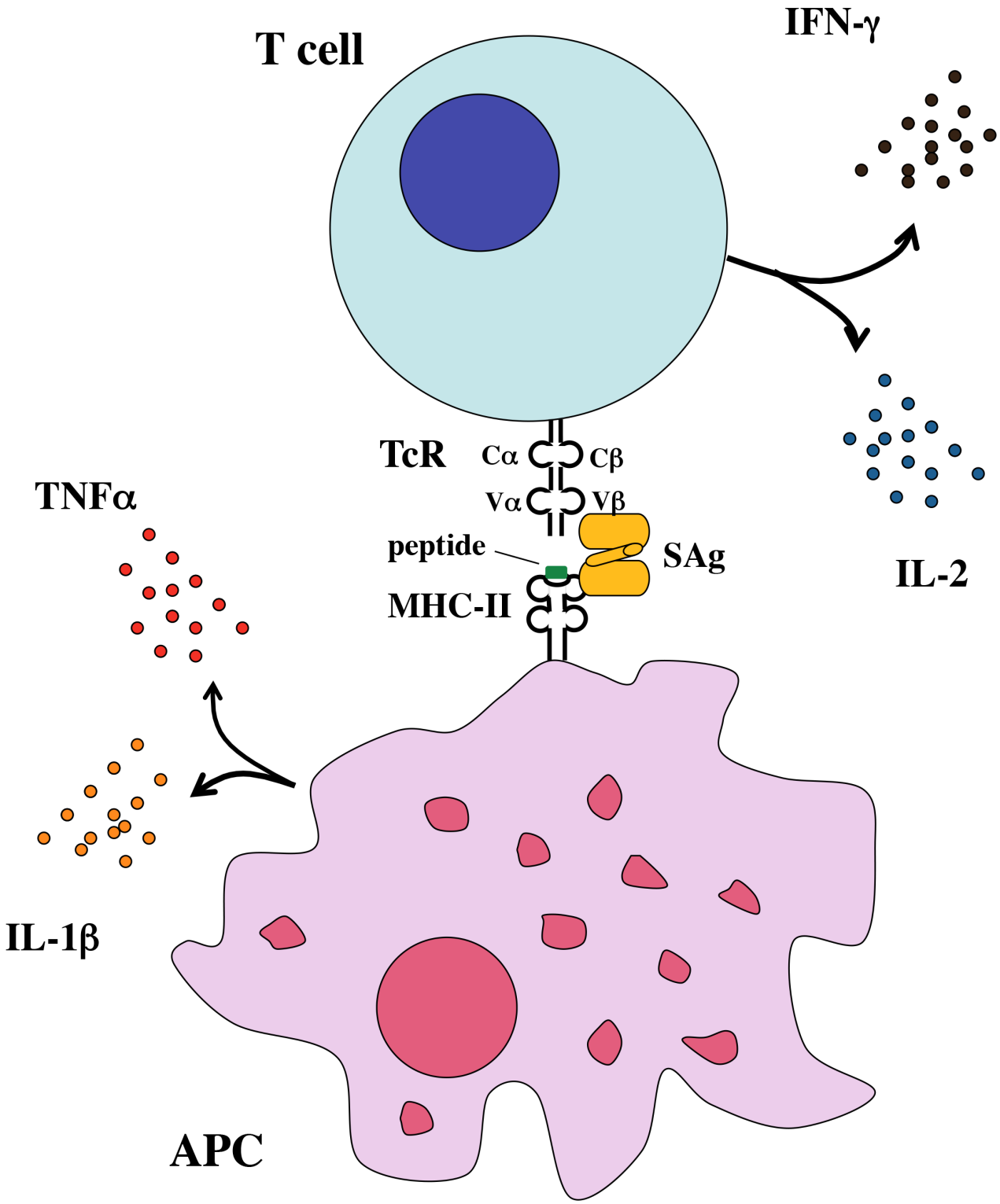

Figure 1. Model of T cell activation by a conventional peptide antigen (Ag) and superantigen (SAg). APC, antigen-presenting cell; MHC II, major histocompatibility class II molecule; TcR, T cell receptor; TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; IL, interleukin.

Table 1. Functional properties of *Streptococcus pyogenes* superantigens

SAg	MW [kDa]	orthologues	alleles	Crystal structure	Zinc binding	MHC II binding α/β chain	Human TcRVβ specificity[f]	P_{50} (h) [pg/ml]	Reference
SPE-A	26.0	Yes[a]	6	+	-	+/-	2.1, 12.2, 14.1, 15.1		(Kim & Watson, 1970; Imanishi, Igarashi, & Uchiyama, 1990; Papagerogiou, et al., 1999; Hartwg, Gerlach, & Fleischer, 1994; Sundberg, et al., 2002)
SPE-C	24.4	Yes[a,b]	2	+	+	-/+	**2.1**, 3.2, 12.5, 15.1	0.1	(Kim & Watson, 1970; Leonard, Lee, Jenkins, & Schlievert, 1991; Roussel, Anderson, Baker, Fraser, & Baker, 1997; Li, Tiedemann, Moffatt, & Fraser, 1997; Sundberg, et al., 2002)
SPE-G	24.6	Yes[a,b]	6	-	+	?/+	**2.1**, 4.1, 6.9, 9.1, 12.3	2	(Proft, Moffatt, Berkahn, & Fraser, 1999)
SPE-H	23.6	Yes[a,c]	2	+	+	-/+	2.1, **7.3,** 9.1, 23.1	50	(Proft, Moffatt, Berkahn, & Fraser, 1999; Arcus, et al., 2000)
SPE-I	26.0	Yes[c]	2	+	+	?/+	6.9, 9.1, **18.1**, 22	0.1	(Proft, Arcus, Handley, Baker, & Fraser, 2001; Brouillard, et al., 2007)
SPE-J	24.6	No	3	+	+	-/+	**2.1**	0.1	(Proft, Arcus, Handley, Baker, & Fraser, 2001; McCormick, Pragman, Stolpa, Leung, & Schlievert, 2001; Baker, et al., 2004)
SPE-K	27.4	Yes[a,c,d]	1	-	+	?/+	**1.1**, 5.1, 23.1	1	(Beres, et al., 2002; Ikebe, et al., 2002; Proft, Webb, Handley, & Fraser, 2003a)
SPE-L	26.2	Yes[a]	3	-	+	?/+	**1.1**, 5.1, 23.1	10	(Proft, Webb, Handley, & Fraser, 2003a; Smoot, et al., 2002a)
SPE-M	25.3	Yes[a,b]	4	+[e]	+	?	**1.1**, 5.1, 23.1		(Smoot, et al., 2002a)
SSA	26.9	No	3	-	-	?	1.1, 3, 15		(Mollick, et al., 1993)
SMEZ1	24.3	No	56	-	+	?/+	2.1, 4.1, 7.3, **8.1**	0.08	(Kamezawa, et al., 1997)
SMEZ2	24.1	No		+	+	?/+	4.1, **8.1**	0.02	(Proft, Moffatt, Berkahn, & Fraser, 1999; Arcus, et al., 2000)

[a] Streptococcus dysgalactiae subsp. equisimilis, [b]Streptococcus dysgalactiae subsp. dysgalactiae, [c]Streptococcus equi subsp. equi, [d]Streptococcus equi subsp. zooepidemicus, [e]Streptococcus dysgalactiae orthologue SPE-M6, [f]principle TcRVβs are in bold and underlined.

The discovery of Group A streptococcal superantigens

Over the last nine decades, eleven SAgs were discovered in *Streptococcus pyogenes* (Figure 2). It all started in 1924 when Dick and colleagues identified a toxin in culture filtrates from hemolytic streptococci isolated from patients with scarlet fever. This toxin was initially named 'scarlet fever toxin' (Dick & Dick, 1983). A second toxin was identified in 1934 and named toxin B (Hooker & Follensby, 1934) followed by the discovery of toxin C from a scarlet fever associated serotype M18 culture filtrate in 1960 (Watson, 1960). The three toxins were immunologically different, but shared several different biological activities, in particular the ability to induce fever when injected into rabbits (pyrogenicity) and the enhancement of susceptibility to endotoxic shock. Based on the strong pyrogenic effect, which was believed to be the primary characteristic of the toxins, Kim and Watson designated the toxins streptococcal pyrogenic exotoxins (SPE) A, B and C (Kim & Watson, 1970). During the 1980s, the toxin genes were cloned and recombinant proteins were produced in *Escherichia coli* and *Bacillus subtilis*, which allowed for a more careful study of the toxin functions in the absence of any contaminating proteins. It was found that SPE-A was identical to Blastogen A, a previously identified T cell mitogen (Schlievert & Gray, 1989), and it was able to activate murine T cells in a MHC class II-dependent and TcRVβ-specific mode (Imanishi, Igarashi, & Uchiyama, 1990). Similarly, the function as a SAg was also established for SPE-C when Leonard and co-workers showed MHC class II and TcRVβ-dependent T cell mitogenicity (Leonard, Lee, Jenkins, & Schlievert, 1991). In contrast, initial findings of SPE-B induced T cell stimulation were later disputed when experiments with recombinant toxin of very high purity could not detect any SAg activity (Gerlach, Reichardt, Fleischer, & Schmidt, 1994). Furthermore, sequencing of the *speB* gene from a serotype M12 *S. pyogenes* strain revealed identity with the gene encoding streptococcal cysteine protease (SCP) (Bohach, Hauser, & Schlievert, 1988).

The identification of SPE-F was reported in 1994 (Norrby-Teglund, Newton, Kotb, Holm, & Norgren, 1994), but it is now believed that the observed mitogenic activity, like in the case of SPE-B, was due to contamination with a powerful SAg. It was later shown that SPE-F is identical with streptococcal DNaseB (Sriskandan, Unnikrishnan, Krausz, & Cohen, 2000).

Musser and colleagues reported in 1993 the discovery of a novel SAg, which they found in the cell culture supernatant of a serotype M3 strain and named streptococcal superantigen (SSA) (Mollick, et al., 1993). Interestingly, SSA showed a higher degree of amino acid similarity to staphylococcal SAgs than to any other streptococcal SAg. Another SAg, called streptococcal mitogenic exotoxin Z (SMEZ), was found in 1997 in the culture supernatant of an M1/T1 *S. pyogenes* strain (Kamezawa, et al., 1997).

SMEZ was the last *S. pyogenes* SAg identified by conventional methods before the start of microbial genomics and the discovery of genes by database mining. The first *S. pyogenes* genome was sequenced from strain SF370, a serotype M1 strain, and raw DNA sequence data was made available on the researchers website at the University of Oklahoma for mining long before completion of the project (Ferretti, et al., 2001). Although the streptococcal (and also staphylococcal) SAgs often share only limited amino acid sequence homologies, they all possess the highly conserved "family signature motifs" Y-G-G-[LIV]-T-X(4)-N (Prosite entry PS00277) and K-X(2)-[LIVF]-X(4)-[LIVF]-D-X(2)-R-X(2)-L-X(5)-[LIV]-Y (PS00278). These motifs were used to mine the SF370 genome database, which resulted in the discovery of four novel *sag* genes, *spe-G, spe-H, spe-I* and *spe-J*. Recombinant forms of the toxins were generated in *E. coli* and functional analysis confirmed their role as SAgs (Proft, Arcus, Handley, Baker, & Fraser, 2001; Proft, Moffatt, Berkahn, & Fraser, 1999). Furthermore, SPE-J was shown to induce fever in rabbits and was lethal in two rabbit models of toxic shock syndrome (McCormick, Pragman, Stolpa, Leung, & Schlievert, 2001). The rapid discovery of novel SAgs by whole genome mining over the following years resulted in an increasingly confusing SAg nomenclature. Analysis of a complete *S. pyogenes* serotype M3 genome in the U. S. resulted in the discovery of a novel *sag* gene that was named *speK* (Beres, et al., 2002). However, this name had already been assigned to an incomplete *sag* gene on the SF370 genome (Ferretti, et al., 2001). In the same year, another group found the same *sag* gene on the genome of a Japanese *S. pyogenes*

serotype M3 strain and named it *speL* (Ikebe, et al., 2002). Shortly after that, this gene was found on a serotype M89 isolate from New Zealand and was also named *speL* (Proft, Webb, Handley, & Fraser, 2003a). It was identified by PCR using specific primers for a previously identified orthologue on a *Streptococcus equi subsp. zooepidemicus* genome. The same strategy also led to the discovery of another novel *sag* gene from a serotype M80 isolate, which was named *speM* (Proft, Webb, Handley, & Fraser, 2003a). At about the same time, two *sag* genes were identified when a serotype M18 isolate genome was completed and named *speL* and *speM* (Smoot, et al., 2002a). *SpeL* is identical to *speM* found on the genome of the M80 isolate, whereas the *speM* gene from the serotype M18 genome had not been reported before. Mitogenic activity of the novel SAgs was confirmed after recombinant proteins were produced in *E. coli* and found to target T cells in a Vβ-specific and MHC class II-dependent mode (Proft, Webb, Handley, & Fraser, 2003a; Smoot, et al., 2002a).

Recently, a novel nomenclature for all streptococcal SAgs was proposed (Commons, et al., 2014). It was suggested to use the name SPE-K for the SAg identified by Beres and co-workers (Beres, et al., 2002), and the names SPE-L and SPE-M for the SAg identified by Smoot and colleagues (Smoot, et al., 2002a). In addition, the names SPE-N, SPE-O and SPE-P were reserved for potential orthologues of the superantigens SzeN, SzeF and SzeP that were recently found in *Streptococcus equi subsp. zooepidemicus* (Paillot, et al., 2010), although these toxins have not been found yet in *S. pyogenes*.

Superantigen orthologues in non-Group A streptococci

Group C Streptococcus (GCS) and Group G Streptococcus (GGS) are commonly regarded as commensals usually found in association with the normal flora of human skin, pharynx and intestine. However, there have been an increasing number of reports implicating GCS and GGS with severe invasive infections, such as necrotizing fasciitis and toxic shock syndrome (Oster & Bisno, 2006). Mitogenic activity in supernatants of clinical GCS and GGS isolates had been reported over several years, but SAgs had not been identified until 2002 when Timoney's group identified two SAgs in *Streptococcus equi*, a bacterium that causes strangles in horses, but can also infect humans.

The *Streptococcus equi* pyrogenic exotoxins H and I (SePE-H, SePE-I) are highly homologous to SPE-H and SPE-I, (>98% amino acid sequence identities) indicating horizontal gene transfer from *S. pyogenes* to *S. equi* or *vice versa* (Artiushin, Timoney, Sheoran, & Muthupalani, 2002). Another two *sag* genes were identified by data mining of the *S. equi* genome at the Sanger Centre and named $speL_{Se}$ and $speM_{Se}$, due to the homology to *speL* and *speM* (recently renamed to *speK* and *speL*, respectively (Commons, et al., 2014)) with 99% and 98.1% nucleotide identities, respectively (Proft *et al.*, 2003b). Two SAgs have been identified from *Streptococcus dysgalactiae* subsp. *equisimilis* called *Streptococcus dysgalactiae*-derived mitogen (SDM) (Miyoshi-Akiyama, et al., 2003) and SPE-G^{dys} (Sachse, et al., 2002). SDM is 99% similar to SPE-M and SPE-G^{dys} is 86% similar to SPE-G.

The recently proposed novel nomenclature for *sag* genes (see above) also included non- *S. pyogenes* SAgs and it was suggested to adapt the names of the *S. pyogenes sag* genes for all orthologues from non- *S. pyogenes sag* genes followed by the allele number (Commons, et al., 2014). For example, *spe-*G^{dys} would be named *speG11* and *sdm* would become *speM6*. Based on this new nomenclature, a search at the National Center for Biotechnology Information Nucleotide (NCBI) database has found *sag* genes in *S. dysgalactiae* subsp. *equisimilis* (3 *speA* alleles, 1 *speC* allele, 16 *speG* alleles, 1 *speH* allele, 3 *speK* alleles, 1 *speL* allele and 1 *speM* allele), S. *dysgalactiae* subsp. *dysgalactiae* (1 *speC* allele, 6 *speG* alleles and 3 *speM* alleles), *S. equi* subsp. *equi* (2 *speH* alleles, 1 *speI* allele and 1 *speK* allele) and *S. equi* subsp. *zooepidemicus* (1 *speK* allele). Interestingly, no orthologues of *ssa*, *smez* or *speJ* were found on any non- *S. pyogenes* genomes. In case of *smez* and *speJ* this is most likely due to the fact that these genes are not associated with mobile DNA elements preventing them from horizontal gene transfer.

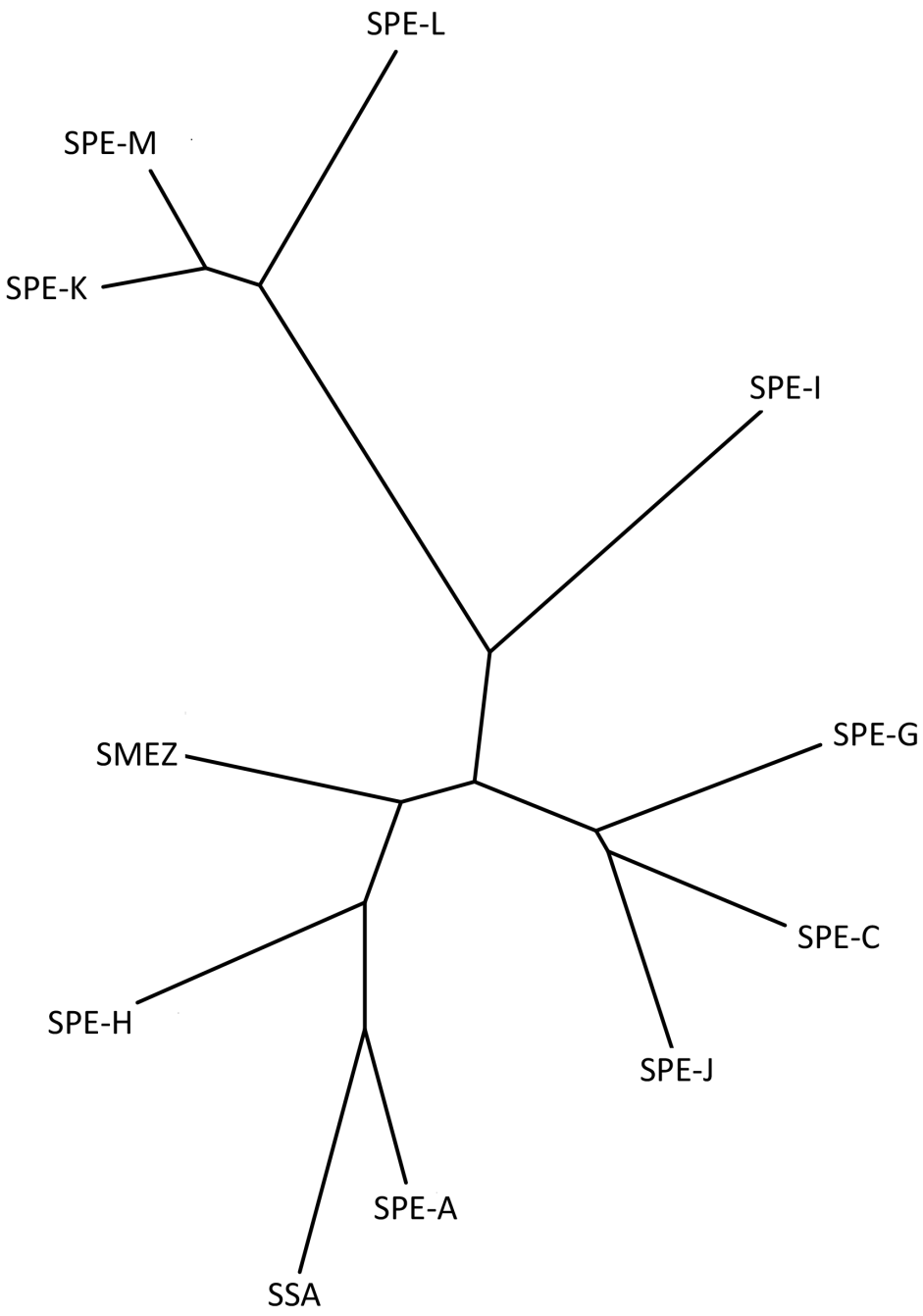

Figure 2. Phylogenetic tree of Group A streptococcal SAgs. The tree was created using ClustalW and is based on primary amino acid sequence homologies of the mature proteins.

Allele diversity and frequency of *sag* genes

In general, *S. pyogenes sag* genes are well conserved and show only minor allelic variation, often not more than just a few nucleic acid differences. The exception is *smez,* of which more than 50 different alleles have been listed in the gene databases. The diversity ranges from single nucleic acid differences to changes in 36 positions (=5%) between *smez-1* and *smez-2*. In addition, several *smez* genes contain nonsense mutations resulting in the expression of truncated and inactive forms of these toxins (Proft, et al., 2000; Turner, et al., 2012). In a recent study, the National Center for Biotechnology Information Nucleotide (NCBI) database was searched for all known streptococcal *sag* gene variants, including genes from non- *S. pyogenes*, and revealed a total of 145 unique alleles belonging to 14 groups. After excluding protein duplicates and truncated variants, a total of 91 unique SAg sequences were identified (Commons, et al., 2014). Currently known SAg variants in *S. pyogenes* are: six SPE-A, three SPE-C, six SPE-G, two SPE-H, two SPE-I, three SPE-J, one SPE-K, three SPE-L, four SPE-M, three SSA, and 56 SMEZ.

S. pyogenes sag genes are generally associated with bacteriophages, with the exception of *speG, speJ* and *smez*, which are chromosomally encoded. However, the *speJ* gene appears to be located on an instable genomic region and is absent in a number of *S. pyogenes* isolates from diverse linages (Friães, Pinto, Silva-Costa, Ramirez, & Melo-Cristino, 2013; Meisal, et al., 2010). It has been suggested that *speJ* has been acquired from a temperate phage that was later lost from the genome of descending *S. pyogenes* lineages (McMillan, et al., 2007). A recent comprehensive profiling of *sag* genes from 480 clinical *S. pyogenes* isolates by multiplex PCR revealed the following distribution: *speA*, 32.1%; *speC*, 51.5%; *speG*, 86.9%; *speH*, 17.1%; *speI*, 15.2%; *speJ*, 32.7%; *speK*, 24.6%; *speL*, 9.2%; *speM*, 9.2%; *ssa*, 35.4%; *smez*, 96% (Friães, Pinto, Silva-Costa, Ramirez, & Melo-Cristino, 2013). The frequencies of individual *sag* genes were generally in agreement with results from previous epidemiological studies (Proft, Webb, Handley, & Fraser, 2003a; Maripuu, Eriksson, & Norgren, 2008; Michaelsen, Andreasson, Langerud, & Caugant, 2011). The *speL* and *speM* genes were detected in only a small fraction of isolates, but were always found together suggesting a stable genetic linkage. Similarly, *speH* and *speI* are relatively rare and were found in association, as expected from their tandem location on a prophage (Commons, et al., 2008), but were also found independently in some isolates from different lineages supporting the idea that *speI* is occasionally lost during phage integration (Proft, Webb, Handley, & Fraser, 2003a; Friães, Pinto, Silva-Costa, Ramirez, & Melo-Cristino, 2013; Maripuu, Eriksson, & Norgren, 2008; Michaelsen, Andreasson, Langerud, & Caugant, 2011).

The origin of *sag* genes is still not entirely clear. The homology between *sag* genes in different streptococcal species, and also in *Staphylococcus aureus*, together with their mainly bacteriophage location suggests horizontal transfer between species. Direct evidence for horizontal gene transfer of a *sag* gene between *S. pyogenes* strains and also between streptococcal species has been provided by Vojtek and co-workers, who showed lysogenic conversion of several *S. pyogenes* M-serotypes and *Streptococcus dysgalactiae* subsp. *equisimilis* clinical isolates with *S. pyogenes* M12-derived prophage phi149 carrying the *ssa* gene (Vojtek, et al., 2008). A recent study has shown that the flanking regions of *speG* in *S. pyogenes* and in *Streptococcus dysgalactiae* subsp. *equisimilis* are conserved suggesting that both species descended from a common ancestor that carried an ancestral *speG* gene (Okumura, et al., 2012).

Regulation of *S. pyogenes* superantigen production

Streptococcus pyogenes SAgs are generally secreted in only small amounts, but little is known about the regulation of these SAgs. In growth medium, gene expression is the highest in the late logarithmic and early stationary phase (Unnikrishnan, Cohen, & Sriskandan, 1999). The production of SMEZ, a potent SAg, is so small that it can only be detected reliably using biological assays involving the detection of T cell mitogenicity (Proft, Sriskandan, Yang, & Fraser, 2003b). There are several lines of evidence showing significant upregulation of *S. pyogenes* SAgs after infection and host factors appear to play a role in this process. SPE-A expression increased

after a diffusion chamber containing *S. pyogenes* was implanted subcutaneously into BALB/c mice. The increase was detected 7 days post-infection and was still high after 21 in vitro passages suggesting a stable switch of the *speA* gene (Kazmi, et al., 2001). The expression of SPE-C could be increased when a *speC*-carrying strain was co-cultured with human pharyngeal cells (Broudy, Pancholi, & Fischetti, 2001). In-vivo up-regulation of SAgs was also shown at the transcription level. In a genome-wide DNA microarray analysis, it was demonstrated that growth of a serotype M1 strain in human blood, compared to growth in growth medium, resulted in significant increase *in speA, speG, speJ* and *smez* transcripts (Graham, et al., 2005). Using the same methodology, *sag* transcription was analyzed during infection of cynomolgus macaques. The *speA, speJ* and *smez* genes were highly expressed in distinct phases of disease. Importantly, *smez* expression was 24-times higher than *speA*, despite the fact that SMEZ is about 10-times more potent in T cell stimulation compared to any other SAg. Furthermore, *smez* expression correlated with peak levels of C-reactive protein (an important inflammation marker) and was the most dominant acute-phase-correlated pro-inflammatory gene. However, there was no correlation of *smez* expression with pharyngitis or tonsillitis suggesting that SMEZ might play an important role in invasive *S. pyogenes* disease (Virtaneva, et al., 2005).

The human factors responsible for SAg upregulation are largely unknown. A study by Kansal *et al.* has shown that expression of SPE-A can be induced by human transferrin and lactoferrin. However, this was not because of a direct effect of these proteins, but rather due to their iron-scavenging activities, as iron deprivation also resulted in increased SPE-A expression, probably due to stress signals (Kansal, Aziz, & Kotb, 2005).

S. pyogenes SAg levels can also be regulated at the protein level. It was shown that SPE-B, a multifunctional cysteine protease is able to degrade SMEZ, whereas SPE-A and SPE-G were more resistant and SPE-J was completely resistant (Nooh, et al., 2006). Interestingly, SPE-B expression is significantly decreased in hypervirulent *S. pyogenes* strains that carry mutations in the two-component CovRS regulator, which suggests that SPE-B might have a role as a global regulator of SAg function through proteolysis (Walker, et al., 2007).

Superantigen protein structure

To date, the protein structures of six *S. pyogenes* SAgs have been solved by X-ray crystallography. These include SPE-A (Papageorgiou, et al., 1999), SPE-C (Roussel, Anderson, Baker, Fraser, & Baker, 1997), SPE-H (Arcus, et al., 2000), SPE-I (Brouillard, et al., 2007), SPE-J (Baker, et al., 2004) and SMEZ-2 (Arcus, et al., 2000). In addition, the protein structure of the *Streptococcus dysgalactiae*-derived mitogen (SDM) has been determined (Saarinen, Kato, Uchiyama, Miyoshi-Akiyama, & Papageorgiou, 2007). SDM shares 92% amino acid identity with SPE-M and has recently been renamed to SPE-M allele 6 (SPE-M6) (Commons, et al., 2014). All protein structures show a conserved two-domain architecture and the presence of a long, solvent-accessible α-helix that spans the center of the SAg molecule, a feature that is shared with the staphylococcal SAgs. The N-terminal domain is a mixed β-barrel with Greek key topology called an oligonucleotide/oligosaccharide binding (OB) fold, which consists of 8 superfamilies, including the 'bacterial enterotoxin' superfamily comprising the 'SAg toxin N-terminal domain' family and the 'bacterial AB_5 toxin' family. Members of the 'SAg toxin N-terminal domain' family also include the staphylococcal SAgs and the superantigen-like toxins (SSLs), which lack mitogenic activity (Arcus, 2002).

The larger C-terminal domain is a β-grasp fold and consists of a twisted β-sheet that is capped by the central α4-helix that packs against a four-strand antiparallel twisted sheet. SAgs are extremely stable proteins that resist denaturing by heat and acid and this is achieved by close packing of the N- and C-terminal domains. The structure is further stabilized by a section of the N-terminus that extends over the top of the C-terminal domain. Notably, the most conserved section of all streptococcal and staphylococcal SAgs, as well as the SSLs, is the region that builds the interface between the α4-helix and the inner side of the N-terminal OB-fold domain.

Molecular interactions of superantigens with host receptor molecules

A hallmark of SAgs is their ability to simultaneously bind to MHC class II molecules on antigen presenting cells and the Vβ-region of the T cell receptor on T cells (Figure 1). A variation in TcR –binding has been shown for the staphylococcal enterotoxin (SE)-H and the *Mycoplasma arthritidis* mitogen (MAM), which both recognize the variable region of the TcR α-chain (Wang, et al., 2007; Petersson, Pettersson, Skartved, Walse, & Forsberg, 2003). However, interaction with TcRVα has not been shown for any of the *S. pyogenes* SAgs. More recently, CD28 has been identified as an additional and essential receptor for cytokine production by both streptococcal and staphylococcal SAgs (Arad, et al., 2011; Kaempfer, Arad, Levy, Hillman, Nasie, & Rotfogel, 2013; Ramachandran, et al., 2013).

MHC class II binding

SAgs have developed a variety of ways for attachment to MHC class II, probably driven by a need to optimize the efficiency of individual SAgs. A very strong and stable binding of SAgs to MHC class II is a prerequisite for the extraordinary amplification in T cell signaling, as less SAg molecules are required for stimulation. This is supported by the fact that about four to five orders of magnitude less SAg molecules are required to stimulate human PBLs compared to mouse PBLs based on a slightly higher affinity towards human MHC class II. Further evidence was provided from experiments with transgenic mice expressing human MHC class II, which are significantly more sensitive to T cell stimulations compared to non-transgenic littermates (Nooh, El-Gengehi, Kansal, David, & Kotb, 2007; Sriskandan, et al., 2001).

In general, streptococcal SAgs bind to MHC class II either via the invariant α-chain or the polymorphic β-chain. The staphylococcal SAgs toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxin (SE)-B are the prototype SAgs for binding to the MHC II α-chain. Co-crystallization studies with these SAgs bound to HLA-DR have revealed an exposed hydrophobic loop region within the N-terminal β-barrel domain that binds to a hydrophobic groove located in the distal region of the DR α1-domain with binding affinities of 10^{-5} M (Jardetzky, et al., 1994; Kim, Urban, Strominger, & Wiley, 1994). This region on the MHC class II molecule has been referred to as the 'generic' or 'low-affinity' binding site for SAgs. Only two of the eleven *S. pyogenes* SAgs, SPE-A and SSA, use this binding mode (Figure 3A). SPE-A competes with SEB for binding to HLA-DR molecules suggesting common recognition sites for MHC class II. However, the binding sites appear to be non-identical, as SPE-A shows higher affinity towards HLA-DQ compared to HLA-DR and HLA-DP, whereas SEB preferentially binds to HLA-DR (Hartwig, Gerlach, & Fleischer, 1994). The other nine *S. pyogenes* SAgs all bind to the MHC class II β-chain via a single, highly conserved histidine residue (His81) in an otherwise highly polymorphic MHC class II molecule. This interaction is based on the formation of a tetravalent zinc complex that includes three residues within the C-terminal domain of the SAgs, also known as the zinc-binding site, in addition to the His81 of MHC class II β-chain. The relative binding affinity of this interaction is about 100-times higher than the generic low affinity site (10^{-7} M) and has been referred to as the 'high-affinity' binding site. SPE-C was the first streptococcal SAg for which this binding mode was shown. SPE-C-binding to the MHC class II β-chain can be completely abolished by adding EDTA and can be restored by excess of Zn^{2+} over EDTA (Li, Tiedemann, Moffatt, & Fraser, 1997). Structural analysis of SPE-C revealed residues His167, His201 and Asp203 as the zinc-binding residues (Roussel, Anderson, Baker, Fraser, & Baker, 1997). The complete zinc coordinated binding was later confirmed by structural analysis of SPE-C in complex with HLA-DR2a bearing a peptide derived from myelin basic protein (Figure 3B). Interestingly, the co-crystal structure also revealed extensive interaction of SPE-C with the bound peptide (Li, et al., 2001). Structural analysis of SPE-H, SPE-J and SMEZ-2, and computer-generated models of SPE-G, SPE-I, SPE-K, SPE-L and SPE-M showed the conserved zinc-binding site in the C-terminal domain, but absence of a generic MHC class II α-chain binding region. This was confirmed in biochemical assays when removal of Zn^{2+} by EDTA completely abolished MHC class II binding.

Interestingly, Scatchard plot analysis of SPE-G, SPE-H, SMEZ and SMEZ-2 revealed a range of different binding affinities (from nanomolar to micromolar) towards MHC class II for each of the toxins. Based on the fact that the generic low-affinity binding site is absent in these toxins and the observation of the extensive SPE-C - peptide interaction, it was suggested that some SAgs might have a more restricted MHC class II repertoire defined by the bound peptide antigen (Proft, Moffatt, Berkahn, & Fraser, 1999). Fernandez and colleagues have shown that despite the important role of the zinc complex in MHC class II binding, about 25% of the contacts are made to the antigenic peptide. However, the interactions are mainly with the peptide backbone atoms rather than the side-chain atoms. Furthermore, SAgs interact with the MHC class II-bound peptides at their conformationally conserved N-terminal regions, minimizing sequence-specific interactions with peptide residues to enhance cross-reactivity (Fernández, Guan, Swaminathan, Malchiodi, & Mariuzza, 2006).

Several SAgs are capable of forming dimers in solution. For example, SPE-C forms a homodimer using a secondary zinc-binding site, which is located within the N-terminal domain. Consequently, the dimer interface is located opposite the high-affinity HLA-DR β-chain binding site and dimer formation might result in DRβ - SPE-C - SPE-C - DRβ complexes (Roussel, Anderson, Baker, Fraser, & Baker, 1997; Li, Tiedemann, Moffatt, & Fraser, 1997). However, zinc-binding and dimerization of SPE-C are not essential for T cell stimulation (Swietnicki, Barnie, Dyas, & Ulrich, 2003). The biological function of SPE-C dimerization is unknown, but one might speculate that MHC class II crosslinking leads to increased expression of co-stimulatory molecules, such as B7, and cell adhesion molecules on antigen presenting cells. It has previously been shown that cross-linking of MHC class II by staphylococcal enterotoxin A (SEA) is necessary for inflammatory cytokine expression (Mehindate, et al., 1995). Dimer formation has also been demonstrated for SPE-J and SSA (Baker, et al., 2004; De Marzí, et al., 2004). However, in both cases the formation of homodimers would prevent the toxins from binding to the TcR. An alternative function, apart from T cell activation has been suggested, but this has never been confirmed.

TcR binding

SAgs bind to TcR molecules primarily by engaging with the variable region of the β-chain (Vβ-domain). This results in an oligoclonal stimulation of a defined T cell repertoire and the potential activation of >20% of all T cells. The first two co-crystal structures of a streptococcal SAg bound to a TcR β-chain were published in 2002 and showed SPE-A in complex with murine TcRVβ8.2 and SPE-C bound to human TcRVβ2.1 (Sundberg, et al., 2002) (Figure 4A). Considering the structural homology between SPE-A and SEB it was not surprising that the SPE-A - mVβ8.2 complex showed strong similarity to the previously solved SEB - mVβ8.2 structure (Li, et al., 1998a). Residues from the complementarity-determining region 2 (CDR2), framework region 2 (FR2) and, to a lesser extent, hypervariable region 4 and FR3 play a role in the interaction of mTcRVβ8.2 with SAgs. Binding to the CDR2 loop appears to be a requirement for all streptococcal and staphylococcal SAgs, whereas binding to other Vβ domains seems to be responsible for Vβ-specificity (Sundberg, Deng, & Mariuzza, 2007). However, there are also some differences in TcR binding of SPE-A compared to SEB. In addition to intermolecular interaction with CDR2, FR3 and hypervariable region 4, SPE-A also binds to the CDR1 loop of the mVβ8 TcR. In addition, there are several hydrogen bonds between SPE-A and mVβ8.2 that involve side chain atoms, whereas the SEB - mVβ8.2 complex shows exclusively main chain contacts.

SPE-C displays a much higher specificity towards T cells targeting mainly TcRVβ2.1 compared to e.g. SPE-A, which binds to Vβ2.1, Vβ12.2, Vβ14.1 and Vβ15.1. This can be explained by a significantly larger buried surface area and the involvement of all Vβ hypervariable loops, including CDR1, CDR2, CDR3, and HV4 (Li, Llera, & Mariuzza, 1998). In addition, residues on the CDR1 and CDR2 loops are involved in extensive intermolecular contacts. Another variation in TcRVβ-binding has been suggested for SPE-I (Figure 4B), which possesses a unique extension (α3-β8 loop) (Brouillard, et al., 2007). A similar extension has been found in the staphylococcal toxins SEI and SEK and the crystal structure of SEK bound to human TcRVβ5.1 has revealed that

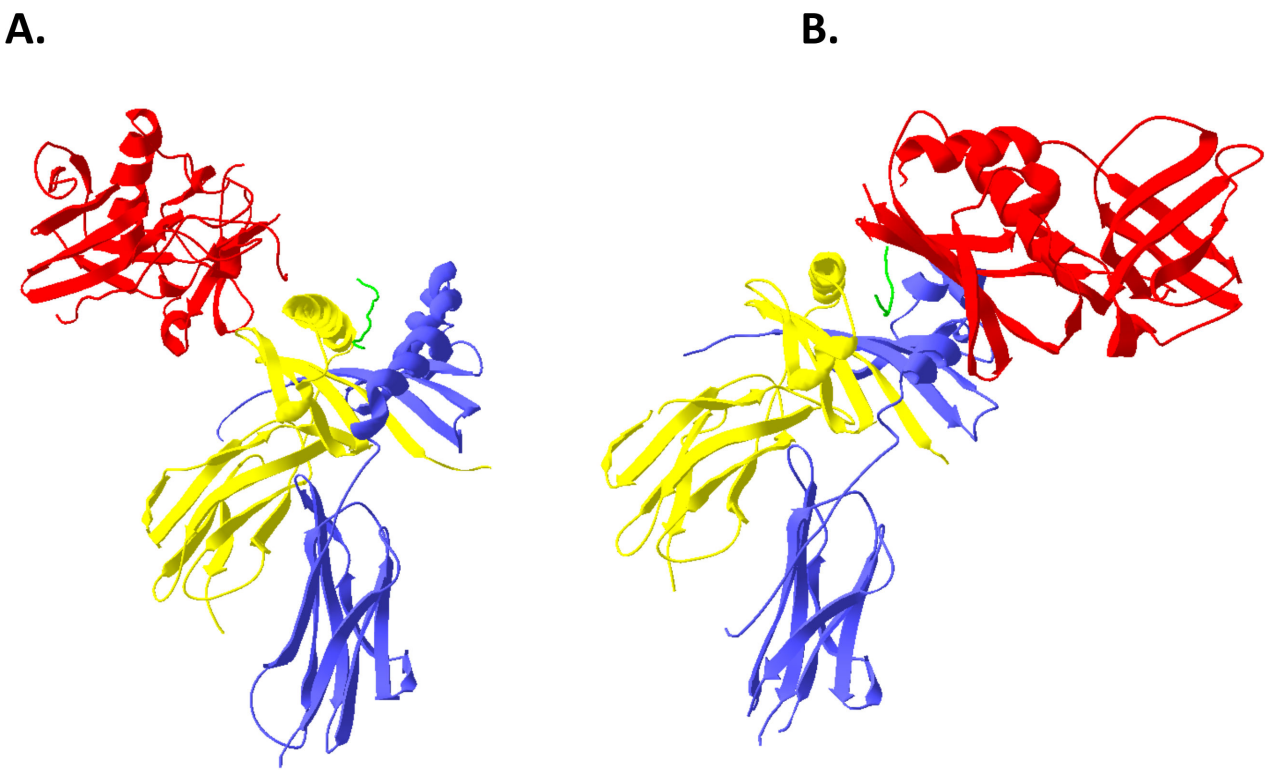

Figure 3. Protein structures of SAg bound to human MHC class II.
A. Structural model of the SPE-A – DR complex in which the SPE-A structure (1B1Z) was superimposed onto the SEB – DR1 structure (1SEB). SPE-A (red) binds to the α-chain of MHC class II (yellow) via the generic "low-affinity binding site" using an exposed hydrophobic loop region within the N-terminal β-barrel domain that binds to a hydrophobic groove located in the distal region of the DR α1-domain.
B. Crystal structure of the SPE-C – DR2 complex (1HQR). SPE-C (red) binds to the polymorphic MHC class II β-chain (blue) with the formation of a tetravalent zinc complex that includes three residues within the C-terminal domain of SPE-C, also known as the zinc-binding site, in addition to the conserved His81 of MHC class II β-chain ("high-affinity binding site"). SPE-C also forms contacts with the bound peptide antigen (green).

residues within the α3-β8 loop make intermolecular contacts with the apical loop of framework region 4 (FR4) (Günther, et al., 2007).

Four categories of SAg - TcR interactions have been proposed: a) highly promiscuous T cell binders, including SEB, that bind to TcRVβ in a simple conformation-dependent mode and only interact with a single CDR2 loop (CDR2); b) moderately promiscuous molecules, including SPE-A, that have direct side chain/side chain contacts in addition to the conformation dependence; c) highly selective T cell activators, like SPE-C, that bind to TcRVβ with the highest degree of structural dissimilarity, and the usage of all three CDR loops (Sundberg, Li, & Mariuzza, 2002) and d) SAgs, like SPE-I, containing the α3-β8 loop and extending the TcRVβ domain binding site into the FR4 region.

Recently, the first ternary complex of a SAg with MHC class II and the TcR was solved. The protein structure of SEB in complex with HLA-DR1 and TcRVα22/Vβ19 confirmed that the SAg adopts a wedge-like position when binding to the TcRVβ-chain, allowing for an interaction between the Vα chain and MHC class II. This binding

mode also circumvents contact between TcR and the presented peptide allowing the SAg to trigger a peptide-independent activation of T cells (Rödström, Elbing, & Lindkvist-Petersson, 2014).

CD28 binding

Over two decades it was believed that the simultaneous interaction of SAg with MHC class II and the TcR was not only necessary, but also sufficient to induce a strong mitogenic activity and the production of large amounts of pro-inflammatory cytokines. This classical view has recently been challenged when it was shown that SAgs can also bind to CD28 (Arad, et al., 2011). CD28 is the general co-stimulatory receptor, which is constitutively expressed on T cells and interacts with B7 molecules (CD80 and CD86) (Riley & June, 2005). It was previously shown that small synthetic peptides mimicking a region that is highly conserved among SAgs (β-strand/hinge/α-helix domain) were strong inhibitors of staphylococcal enterotoxin B (SEB), staphylococcal toxic-shock syndrome toxin-1 (TSST-1), and SPE-A and were protective in mice against a lethal challenge with those SAgs. Notably, the peptide region was neither involved in MHC class II binding, nor in binding to TcR. In contrast, synthetic peptides of regions known to interact with MHC class II or TcR failed to reduce a cytokine response (Arad, Levy, Hillman, & Kaempfer, 2000). It was later shown that the synthetic peptide successfully competed with a monoclonal anti-CD28 antibody for binding to CD28 without directly binding to the antibody. This suggested an interaction of the peptide with CD28. Direct binding of the peptide and of staphylococcal enterotoxin B to CD28 with micromolar affinity was demonstrated by surface plasmon-resonance analysis and this interaction is essential for the induction of pro-inflammatory cytokine genes (Arad, et al., 2011). A structural model was suggested that shows a possible binding interface between the N-terminal 118-residue region of the extracellular domain of CD28 (1yjd) with a freely accessible β-strand/hinge/α-helix domain of staphylococcal enterotoxin C3 (SEC3) in complex with MHC class II α-chain and mTcRVβ8.2 (1jck) (Arad, et al., 2011). More recently, it was demonstrated that a CD28 mimetic peptide protects mice from a lethal challenge with SPE-A, as well as from a lethal *S. pyogenes* infection in a mouse necrotizing soft tissue infection model providing further evidence for the importance of a SAg-CD28 interaction in SAg-mediated disease (Ramachandran, et al., 2013).

It should be mentioned that all the research described above was carried out with SAgs that bind to the MHC class II α-chain. However, nine of the eleven SAgs produced by *S. pyogenes* bind to the MHC class II β-chain via the zinc-binding site in the C-terminal domain and this binding mode would sterically hinder the suggested interaction with CD28. Therefore, it is currently unclear, if the activity of those SAgs is CD28-independent, or if there is another binding site for CD28.

Consequences of SAg binding to host receptors

Engagement of SAg with its receptors results in rapid release of TNF-α and TNF-β, followed sequentially by IL-2, IL-6, IL-1 and IFN-γ. Animal studies with mice have shown a dramatic increase of TNF-α within the first hour of SAg exposure and T cells within the spleen were found to be the major source for the early release of TNF-α (Faulkner, Cooper, Fantino, Altmann, & Sriskandan, 2005). A comparative study with SPE-A and SMEZ showed that the cytokine-inducing capacity of SMEZ was approximately 10-fold higher than observed with SPE-A (Müller-Alouf, et al., 2001). Furthermore, disruption of the *smez* gene in an M89 strain completely abolished cytokine production of a *S. pyogenes* culture supernatant in vitro (Unnikrishnan, et al., 2002).

Early studies have shown that a combination of sub-lethal doses of SAg and LPS can act synergistically to cause shock in rodents, although only when D-galactosamine was used as a sensitizing agent (Bohach, Fasdt, Nelson, & Schlievert, 1990). Similarly, primary human monocytes that were pre-exposed to SAgs for 3 hours showed highly exaggerated TNF-α responses after exposure to LPS. It has been suggested that this synergy results from enhanced pattern recognition of LPS and this is based on the observation that SAg signaling increases expression of toll-like receptor 4 (TLR4), the pattern recognition receptor for LPS (Hopkins, et al., 2005).

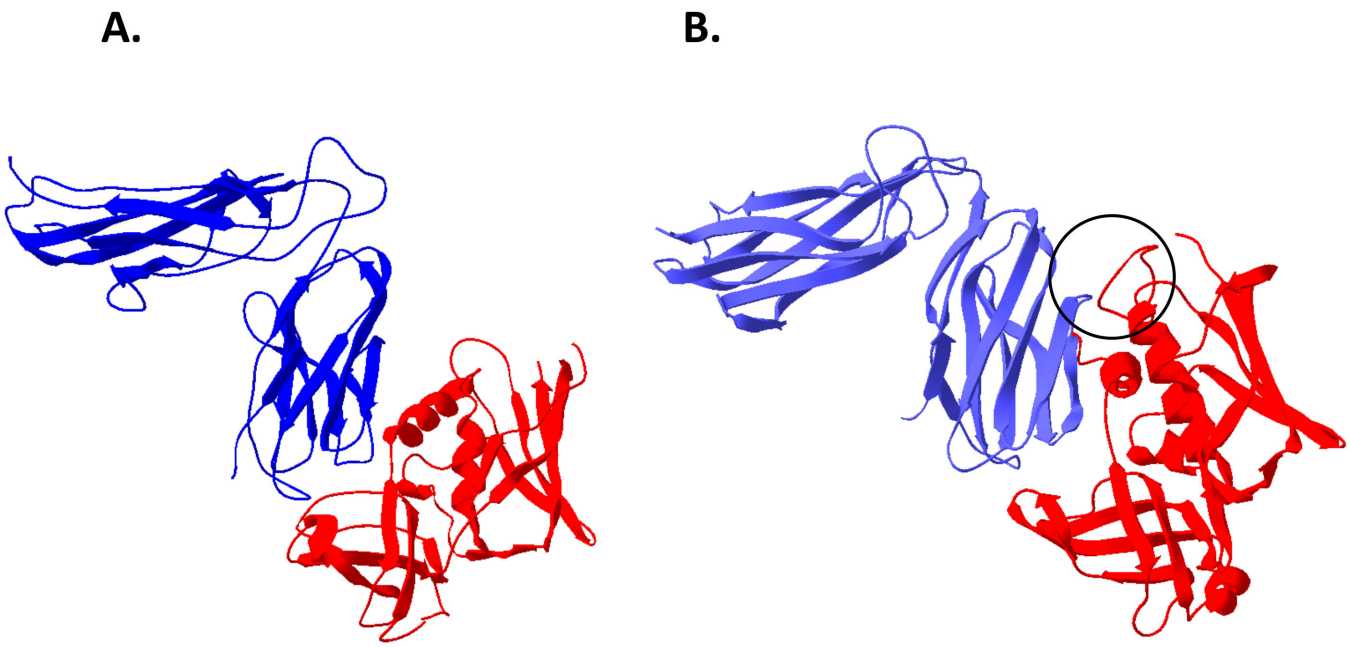

Figure 4. Protein structures of SAgs bound to the T cell receptor.
A. Crystal structure of SPE-C bound to the human TcRVβ2 chain (1KTK). SPE-C (red) shows high specificity for hVβ2.1 (blue) due to extensive interactions involving all hypervariable loops (CDR1, CDR2, CDR3, and HV4).
B. Structural model of SPE-I in complex with hTcRVβ5.1 in which the SPE-I structure (2ICI) was superimposed onto the SEK – hTcRVβ5.1 structure (2NTS). Like SEK, SPE-I (red) has a unique α3-β8 loop that forms intermolecular contacts with the apical loop of framework region 4 (FR4) of the TcRVβ chain (blue) (shown in circle).

Furthermore, several SAgs, including the streptococcal SAgs SPE-A and SMEZ, were able to up-regulate TLR2 on the surface of primary human monocytes. This was dependent on SAg-binding to MHC class II, but did not involve signaling by ligation to TLR2. TLR2 up-regulation was associated with an increase in the pro-inflammatory response to TLR2 ligands, but only at high ligand concentration (Hopkins, et al., 2008).

In contrast to the classical AB family toxins, SAgs are believed to remain extracellular and function by signaling inside the host cell. However, a recent study by Ganem *et al.* has demonstrated an uptake of SAgs by mouse dendritic cells (DCs) without triggering DC maturation. This was followed by SAg recycling to the cell membrane of DCs and the SAg-loaded DCs were capable of triggering a strong lymphocyte proliferation. The authors suggested that intracellular trafficking of SAgs might increase the local concentrations of SAgs and promote their encounter with MHC class II on APCs and the TcR on T cells in lymph nodes (Ganem, et al., 2013).

Streptococcus pyogenes superantigens and disease

SAgs have been implicated in a range of *S. pyogenes* diseases, including invasive infections such as necrotizing fasciitis and streptococcal toxic shock syndrome (STSS), Kawasaki disease, psoriasis and acute rheumatic fever. The potential involvement of SAgs in these diseases has been demonstrated mainly by epidemiological studies, clinical studies and animal infections models. In addition, several studies have shown specific skewing of the TcRVβ-repertoire in stimulated T cells consistent with SAg activity. However, direct evidence for the involvement of SAgs in *S. pyogenes* disease remains inconclusive.

Invasive *Streptococcus pyogenes* disease

Epidemiological studies

The predominant strains isolated from patients with STSS belong to serotype M1 and M3 which both frequently produce SPE-A and SPE-C (Talkington, et al., 1993; Yu & Ferretti, 1989). This association was also found in several other epidemiological studies. The *speA* gene was found in a majority (40-90%) of *S. pyogenes* isolates from the USA associated with invasive disease and STSS, but only in a minority (15-20%) of isolates from non-invasive diseases (Hauser, Stevens, Kaplan, & Schlievert, 1991). Cleary and colleagues reported *speA*-carrying isolates in 90% of 17 isolates causing sepsis, but in only 54% of 37 isolates that caused non-invasive disease (Cleary, et al., 1992). A high frequency of *speA* (80%) was found in STSS isolates collected in Australia (Carapetis, Robins-Browne, Martin, Shelby-James, & Hogg, 1995) and of 53 STSS isolates from Europe and Chile, 64% carried the *speA* gene and 28% carried *speC* (Reichardt, Müller-Alouf, Alouf, & Köhler, 1992). Vlaminckx *et al.* analyzed 170 *S. pyogenes* isolates that caused specific manifestations of invasive disease in The Netherlands between 1992 and 1996. They found a strong correlation of a M1 clone carrying *speA* and *smez* with toxic shock-like syndrome. Furthermore, *S. pyogenes* isolates carrying the *speC* gene were found predominantly in patients with invasive disease not accompanied with streptococcal toxic shock syndrome. The authors also established associations of *speA* with meningitis, *speH* with arthritis and *speC* with puerperal sepsis (Vlaminckx, et al., 2003). In a more recent study conducted in Norway, *speA* was identified in 41% of 22 invasive isolates, but only in 11% of 101 non-invasive isolates (Kittang, Skrede, Langeland, Haanshuus, & Mylvaganam, 2011). Interestingly, a worldwide shift in *speA* alleles has occurred over the past 80 years. Contemporary M1 and M3 strains almost exclusively harbor *speA2* and *speA3*, respectively, and these alleles have been associated with the re-emergence of invasive infections with more virulent *S. pyogenes* strains. A more recent study that analyzed the genome sequences from 3,615 M1/*emm1* strains from different locations between 1920 and 2013 suggests that acquisition of the *speA* gene was an important step in the evolution of a hypervirulent M1/*emm1* strain. It appears that an early M1/*emm1* strain acquired a plasmid carrying the *speA1* allele, which subsequently evolved into the *speA2* allele. Acquisition of a large chromosomal region carrying genes for additional virulence factors (Streptolysin O and NAD^+-glycohydrolase) was the final molecular event preceding the emergence of the hypervirulent M1/*emm1* strain in the 1980s (Nasser, et al., 2014).

However, there are also reports that showed no significant difference in the frequency of *speA* between invasive and non-invasive *S. pyogenes* isolate (Descheemaeker, Van Loock, Hauchecorne, Vandamme, & Goossens, 2000; Haukness, et al., 2002; Hsueh, et al., 1998; Mylvaganam, Bjorvatn, & Osland, 2000). For example, Haukness and co-workers compared the genetic heterogeneity of 63 community pediatric pharyngeal isolates with 17 contemporaneous invasive pediatric isolates and found that more pharyngeal (71%) than invasive isolates (35%) were positive for both *speA* and *speC* (Haukness, et al., 2002).

An association with invasive disease was also reported for other SAgs. An invasive M3/T3 strain emerged during the 1990' in Japan and 100% of 18 isolates carried the phage-encoded *speK* gene (formerly *speL*). In contrast, none of the 10 non-invasive isolates collected before 1992 harbored the *speK* gene (Ikebe, et al., 2002). In another Japanese study with isolates collected between 1994 and 1999, *ssa* was detected in 76% of 17 invasive isolates, but

only in 37% of 299 non-invasive isolates (Murakami, et al., 2002). A recent study compared a collection of 160 isolates recovered from normally sterile sites with 320 isolates associated with pharyngitis in Portugal and observed an association of *speJ* with invasive *S. pyogenes* isolates (Friães, Pinto, Silva-Costa, Ramirez, & Melo-Cristino, 2012).

An association of the *speM* gene with invasive disease was suggested after a study with *S. pyogenes* isolates collected in Germany between 1997 and 2003 showed that *speM* was more commonly found in invasive disease isolates compared to non-invasive isolates (Lintges, et al., 2010).

The *smez* gene is chromosomally encoded and found in almost all *S. pyogenes* isolates. Therefore, there is no association of this toxin gene with invasive disease. However, *smez* is the most variable of all *sag* genes and there are more than 50 *smez* alleles listed in the NCBI database. Furthermore, *smez* alleles are in linkage equilibrium with *S. pyogenes* M-serotypes and there are significant differences in mitogenic potencies between SMEZ variants (Proft, et al., 2000). However, no studies have analyzed a possible correlation of certain *smez* alleles with invasive disease. Notably, it has recently been discovered that the STSS-associated *emm3* strain carries a *smez* variant with a 13-bp deletion that causes a frame-shift and consequently disrupts SAg activity (Turner, et al., 2012).

Clinical Studies

SPE-A was detected in the sera of two patients with STSS using immunoassays. The presence correlated with elevated levels of TNF-α, providing evidence of SPE-A-induced T cell activation (Sriskandan, Moyes, & Cohen, 1996). Strong mitogenic activities were found in the serum of two patients with STSS, one of whom died. PCR-analysis of the infecting *S. pyogenes* isolates identified the presence of several *sag* genes, including *speA*, *speC, speG, speJ* and *smez.* Using a T cell proliferation assay with recombinant protein standards, the mitogenic activity in the serum could be wholly attributed to *smez*, with a small contribution of *speJ* in one case, and the concentration of the circulating SAg was approximately 100 pg/ml. Furthermore, analysis of the convalescent serum from the surviving patient showed sero-conversion to SMEZ, providing further evidence for the involvement of SMEZ in STSS (Proft, Sriskandan, Yang, & Fraser, 2003b). It has been suggested that the lack of neutralizing antibodies against SAgs might be a risk factor in invasive disease and supportive evidence was provided by several other studies. Eriksson and co-workers showed that sera from STSS patients did not neutralize SPE-A-induced lymphocyte mitogenicity and neutralization was low in patients with bacteremia compared with serum levels from uncomplicated erysipelas (Eriksson, Andersson, Holm, & Norgren, 1999). A study by Basma *et al.* found significantly higher plasma levels of neutralizing anti-SPE-A antibodies in patients with severe and non-severe invasive *S. pyogenes* disease compared to age- and geographically matched healthy donors (Basma, et al., 1999). In a case study of a patient with STSS from New Zealand, an *emm118* strain was isolated from the patient and the major mitogenic toxin produced by this isolate was identified as SMEZ-34, which is closely related to the highly potent SMEZ-2 variant. No neutralizing anti-SMEZ-34 antibodies could be detected in the acute serum, but were found in convalescent serum (Yang, et al., 2005).

Animal Infection Models

A baboon model of *S. pyogenes* bacteremia that mimics human STSS was used to demonstrate the in-vivo effect of SAgs. Intravenous infusion of a SPE-A-expressing M3 strain led to profound hypertension leukopenia, metabolic acidosis, renal impairment, thrombocytopenia and disseminated coagulopathy within 3 hours (Stevens, et al., 1996). In another study, a murine model of bacteremia and *S. pyogenes* muscle infection was used to investigate the role of SPE-A. Surprisingly, infection with a *speA* deletion mutant failed to attenuate virulence, but instead resulted in increased bacteremia and a reduction of neutrophils at the site of infection (Sriskandan, et al., 1996b). It was suggested that the reduced binding affinity of SAgs to murine MHC class II molecules might be reason for the unexpected result. Indeed, the use of HLA-DQ transgenic mice rendered the animals susceptible to SPE-A and resulted in massive cytokine production and lethal shock (Sriskandan, et al., 2001). The

same HLA-DQ transgenic mouse model was also used to assess the role of SMEZ in disease. Intraperitoneal infection of the animals with a M89 strain expressing the SMEZ-13 variant resulted in significantly increased cytokine production. In contrast, infection with an isogenic M89 *smez* deletion mutant failed to elicit a response, despite the fact that this *S. pyogenes* isolate also carried other *sag* genes suggesting an important role for SMEZ in invasive disease (Unnikrishnan, et al., 2002). The in vivo role of SAgs was also shown in rabbit infection models with SPE-A (Schlievert, Assimacopoulos, & Cleary, 1996) and SPE-J, which induced fevers and was lethal in two models of STSS (McCormick, Pragman, Stolpa, Leung, & Schlievert, 2001).

Studies based on changing T cell repertoires

Stimulation with SAgs leads to an initial TcRVβ-restricted proliferation of T cells followed by the loss of the particular T cell subsets due to anergy leaving a kind of 'fingerprint'. Michie and colleagues collected two *S. pyogenes* isolates from two patients with STSS which both produced a mitogen specific for the Vβ2 T lymphocyte subset in vitro. Lymphocytes collected from both patients during the acute phase demonstrated a marked reduction in circulating 'naive' and helper T cells expressing Vβ2, and an increase of CD8 T cells expressing Vβ2 (Michie, Scott, Cheesbrough, Beverley, & Pasvol, 1994). Another study compared the TcRVβ repertoire in T cells from variety of disease patients and found a consistent pattern of depletion of Vβ1, Vβ5.1, and Vβ12 in patients with severe *S. pyogenes* infections, but not in patients with non-severe infections or patients with severe no *S. pyogenes* infections (Watanabe-Ohnishi, et al., 1995). Yet another study reported the expansion of TcRVβ2 T cells from two patients with STSS reflecting the production of SPE-C (Thomas, et al., 2009).

Genetic background of the host

The results from several studies suggest that the genetic background of the host, in particular HLA polymorphism, might play an important role in invasive disease susceptibility. SPE-A was shown to stimulate higher proliferation responses when presented by HLA-DQ, compared to HLA-DR1, HLA-DR4, or HLA-DR5 alleles, whereas SPE-C was preferentially presented by HLA-DR4 (Norrby-Teglund, Nepom, & Kotb, 2002). Moreover, patients with the HLA-DRB1*1501/DQB1*0602 haplotype showed significantly reduced responses to streptococcal SAgs and were less likely to develop severe systemic disease compared to individuals with risk or neutral haplotypes (Kotb, et al., 2002). Llewelyn and colleagues reported a stronger affinity of SPE-A for HLA-DQA1*01 compared to HLA-DQA1*03/05, which also resulted in quantitative and qualitative differences in T cell proliferation, cytokine production, and TcRVβ-specific changes in the T cell repertoire (Llewelyn, et al., 2004). In contrast, a study using HLA-DQ transgenic mice found that HLA-DQ6 and HLA-DQ8 elicited comparable in vitro and in vivo immune response to SPE-A, SPE-C and SMEZ (Rajagopalan, et al., 2008).

A more recent study showed that HLA alleles not only influenced the severity of SAg-mediated disease, but also effected the polarization of the cytokine response. In contrast to the high-risk alleles HLA-DR14/DR7/DQ5, HLA-DR15/DQ6 alleles strongly protected against severe invasive *S. pyogenes* disease and elicited significantly higher amounts of anti-inflammatory cytokines, such as IL-10, compared to pro-inflammatory cytokines, like IFN-γ (Nooh, Nookala, Kansal, & Kotb, 2011).

Kawasaki Disease

Kawasaki disease (KD) is an acute multisystem vasculitis of unknown etiology that affects mostly young children leading to coronary artery damage (Takahashi, Oharaseki, & Yokouchi, 2014). Streptococcal SAgs have been proposed as etiological agents in the pathogenesis of KD. Multiple studies reported the selective expansion of T cells bearing TcRVβ2 pointing to a possible involvement of SAgs in the disease, in particular SPE-C and SPE-J which both preferentially stimulate the TcRVβ2 T cell subset (Abe, et al., 1992; Konishi, et al., 1997). Yoshioka *et al.* reported polyclonal expansion of TcRVβ2- and TcRVβ6-bearing T cells and elevated plasma levels of IL-1β, IL-2, IL-6, IL-8, IL-10, IFN-γ, and TNF-α in the acute phase of KD. Moreover, anti-SPE-C antibody levels were significantly higher in acute and convalescent serum from KD patients compared to age-matched controls

(Yoshioka, et al., 1999). High levels of anti-SPE-A IgM antibodies were also found in KD patients and increased with the clinical weeks reaching 43% of KD subjects at the fourth week (Matsubara, et al., 2006).

PCR-analysis of *speA, speC, speG*, and *speJ*, in stool specimen obtained from 60 patients with KD and 62 age-matched children showed higher prevalence of *sag* genes in KD patients compared to controls (Suenaga, Suzuki, Shibuta, Takeuchi, & Yoshikawa, 2009). Two studies that investigated the T cell repertoire in KD patients also provided evidence for a role of SAgs in KD. TcRVβ restricted CD4 and/or CD8 activation was observed in eight of 11 (72%) of the KD patients, a finding not observed in healthy controls. Moreover, 81% children with KD had evidence of either TcRVβ skewing (particularly CD4 Vβ2 and Vβ5.1) and/or TcRVβ restricted activation (Brogan, Shah, Clarke, Dillon, & Klein, 2008). Nagata and colleagues identified 18 strains of Gram-positive cocci from the upper gastrointestinal tract from patients with KD that had superantigenic properties and which induced the expansion of TcRVβ2 T cells in vitro (Nagata, et al., 2009).

However, other investigations have failed to show any evidence for SAg involvement in KD. In particular, data from several serological studies showed no significant difference in the prevalence of SAg antibodies between KD patients and control subjects (Gupta-Malhotra, Viteri-Jackson, Thomas, & Zabriskie, 2004; Morita, Imada, Igarashi, & Yutsudo, 1997; Nomura, Yoshinaga, Masuda, Takei, & Miyata, 2002). Furthermore, IgM transcripts expressed by the B cells in the peripheral blood of KD patients in the acute phase of the disease clearly showed an oligoclonal expansion, suggesting that KD is caused not by stimulation of a SAg, but rather by a conventional antigen (Lee, Shin, Kim, & Park, 2009).

Psoriasis

Psoriasis is a chronic inflammatory multi organ disease with well-characterized pathology occurring in the skin and often the joints (Raychaudhuri, Maverakis, & Raychaudhuri, 2014). It has been reported that a particular form of psoriasis, guttate psoriasis, is triggered by *S. pyogenes* throat infections in 2/3 patients (Nahary, et al., 2008). The causes of psoriasis are not fully understood, but several lines of evidence point to an involvement of SAgs in the disease mechanism. Some studies have demonstrated a TcRVβ-restricted T cell stimulation in psoriasis patients. Leung and co-workers have shown T cell expansion consistent with SAg activity in skin biopsies from two patients with psoriasis, but not in peripheral blood. Skin biopsies from 10 out of 10 patients with acute guttate psoriasis, but not skin biopsies from 12 patients with acute atopic dermatitis or inflammatory skin lesions induced in normal subjects with sodium lauryl sulfate, demonstrated selective accumulation of TcRVβ2 T cells, which occurred in both the CD4+ and the CD8+ T cell subsets. Moreover, the TcR showed extensive junctional region diversity suggesting SAg-induced stimulation of T cells (Leung, et al., 1995). Other studies demonstrated an increase of TcRVβ2 and Vβ5.1 T cells in the skin of patients with guttate and chronic plaque psoriasis compared with peripheral blood (Lewis, et al., 1993) and an increase of TcRVβ2 and Vβ17 cutaneous T cells in patients with guttate psoriasis, but not in control patients (Davison, Allen, Mallon, & Barker, 2001). On the other hand, since 1994, at least 14 studies reported by nine independent groups have indicated that chronic psoriasis lesions are infiltrated by oligoclonal T cells suggesting stimulation by conventional antigens rather than SAgs (reviewed by (Valdimarsson, Thorleifsdottir, Sigurdardottir, Gudjonsson, & Johnston, 2009).

Acute Rheumatic Fever

Acute rheumatic fever (ARF) is a post-streptococcal autoimmune disease. Multiple episodes can result in rheumatic heart disease (RHD), which is the leading cause of preventable pediatric heart disease. It mainly occurs in school age children and young adults after pharyngeal infection with *S. pyogenes* (Carapetis, Steer, Mulholland, & Weber, 2005). Cross-reactive immune responses to cardiac tissue and joints are responsible for inflammation in the host and it has been suggested that SAgs might stimulate the reactive T cells. There is a correlation between M18 isolates associated with ARF in the USA and *speL* and *speM*. These *sag* genes were

found in all M18/emm18 isolates collected over a 69-year period (Smoot, et al., 2002b). Antibodies against SPE-L and SPE-M were more common in convalescent sera from ARF patients compared to pharyngitis patients (Smoot, et al., 2002a). However, serum antibodies against SAgs did not predict the susceptibility of Aboriginal Australians (Yang, et al., 2006).

Therapeutic Interventions

Intravenous immunoglobulin (IVIG) therapy

Pooled human intravenous immunoglobulin (IVIG) is increasingly used in cases of severe invasive *S. pyogenes* disease to neutralize the activity of SAgs. Several studies have shown that the lack of protective antibodies against SAgs is a risk factor for toxic shock syndrome (Eriksson, Andersson, Holm, & Norgren, 1999; Basma, et al., 1999; Norrby-Teglund, Low, & Kotb, 2007). IVIG were used with bacterial culture supernatants and showed good neutralization properties, in particular against streptococcal SAgs (Darenberg, Söderquist, Normark, & Norrby-Teglund, 2004). The clinical efficacy of IVIG in STSS was documented in several case reports, two observational cohort studies, one case-control study and one multicenter placebo-controlled trial (Norrby-Teglund, Low, & Kotb, 2007). However, definitive clinical trial data are still lacking. Several factors need to be considered in the use of IVIG as adjunctive STSS therapy. SAgs appear to have a very fast turnover rate in the patient's blood and might therefore only be beneficial if applied very early after the onset of the disease. Secondly, not much is known about SAg expression during infection. It has been shown in animal infection models that SAgs are up-regulated significantly during disease (Kazmi, et al., 2001; Virtaneva, et al., 2005). Finally, the efficacy of IVIG to neutralize streptococcal SAgs was shown to vary between different preparations of IVIG (Schrage, Duan, Yang, Fraser, & Proft, 2006).

Peptide antagonists

Kaempfer and colleagues synthesized several short peptides derived from various SEB domains and found a dodecapeptide that weakly antagonized SEB activity. A modified version of this peptide was shown to be a more powerful antagonist that inhibited the activity of SEB and TSST-1 in a mouse infection model (Arad, Levy, Hillman, & Kaempfer, 2000). It was originally unclear how this peptide, which is highly conserved in both streptococcal and staphylococcal SAgs would work, as the SEB domain from where the peptide is derived is not involved in either MHC class II or TcR binding. More recently, it was shown that the peptide binds to the co-stimulatory receptor CD28 and this interaction is essential for the induction of pro-inflammatory cytokine genes (Arad, et al., 2011). More recently, it was demonstrated that the CD28 mimetic peptide AB103 protects mice from a lethal challenge with SPE-A, as well as from a lethal *S. pyogenes* infection in a mouse necrotizing soft tissue infection model (Ramachandran, et al., 2013).

Receptor mimics

A bispecific receptor mimic that targets both the MHC class II and the TcR binding site of SAgs was designed by Lehnert and co-workers. This construct consists of a HLA-DR1α1 subunit that is connected to the variable TcR β-chain via a peptide linker. The authors generated several different receptor mimics, each one specific against a particular SAg. For example, human TcRVβ3 was used for a SEB-specific molecule, human TcRVβ2 was used for a TSST-1-specific chimera, and an analogue of murine TcRVβ8.2 was used for SEC3-specific chimera (Lehnert, et al., 2001). In a cell proliferation assay, 20-times excess of the TcRVβ8.2 chimera over SEC3 showed 40% inhibition.

The efficacy of TcR antagonist was later improved by using yeast display libraries of random and site-directed human TcRVβ8 mutants to screen for improved domain stability and increased SAg binding (Buonpane, et al., 2007). A panel of six soluble, high-affinity TcRVβ mutants have been engineered that bind to one of six key staphylococcal and streptococcal SAgs (SEA, SEB, SEC3, TSST1, SPE-A, and SPE-C), at the same epitope as the

wild type receptors. Affinities were in the picomolar to nanomolar range representing 1000 to 3,000,000-fold increases, compared to wild-type (Sharma, Wang, & Kranz, 2014; Wang, Mattis, Sundberg, Schlievert, & Kranz, 2010).

Toxoid vaccines of SAgs

Toxoids of two streptococcal SAgs were generated by Schlievert and colleagues. Double-, triple- and hexa-amino acid mutants of SPE-A targeting MHC class II and TcR binding sites lacked SAg activity, were non-lethal in two rabbit models of STSS and stimulated protective antibody responses (Roggiani, et al., 2000). Similarly, the SPE-C Y15A/N38D double mutant and the SPE-C Y15A/H35A/N38D triple mutant were non-mitogenic, non-lethal in rabbit models of STSS and protected vaccinated animals from challenge with wild-type SPE-C (McCormick, et al., 2000).

SAgs as vaccine conjugates

By triggering MHC class II signals without engaging with the TcR, SAgs might be excellent vaccine adjuvants of the innate immune response due to their priming effects on antigen presenting cells (Hopkins, et al., 2005). In a recent study, the TcR-binding site of SMEZ-2 was mutated by converting three amino acid residues, W75L, K182Q, and D42C. The cysteine at position 42 was introduced to allow for easy coupling with desired peptides. The T cell proliferation response of the mutant (SMEZ-2-M1) was $>10^5$-fold lower compared to wild-type and cytokine production in response to the mutant was undetectable. Vaccination of mice with ovalbumin conjugated to SMEZ-2-M1 resulted in anti-ovalbumin IgG titers being 1,000-10,000-fold higher than in mice immunized with unconjugated ovalbumin (Radcliff, et al., 2012). Conjugating antigens to SMEZ-2-M1 also increased the efficiency for cross-presentation. When co-injected with an adjuvant, the SMEZ-2-M1 conjugates also elicited potent T cell responses with antitumor activity (Dickgreber, et al., 2009). More recently, it was demonstrated that dendritic cells pulsed with the nucleocapsid of hepatitis B virus conjugated to SMEZ-2-M1 (M1:HBcAgs) stimulated virus-specific CD(8+) T cells more effectively than dendritic cells pulsed with native virus capsid, which also suggests that SMEZ-2-M1 conjugates increase cross-presentation by APCs (McIntosh, et al., 2014).

In another study, SMEZ-2-M1 conjugated with myelin oligodendrocyte glycoprotein 35-55 peptide suppressed the development of experimental autoimmune encephalomyelitis (EAE) in mice via antigen-specific suppression of T cell responses and re-establishing of suppressor function of Ly6G(-)CD11b(+) blood monocytes (Slaney, Toker, Fraser, Harper, & Bäckström, 2013). These studies suggest a potential use of SMEZ-2-M1 as antigen carrier for vaccination, anti-tumor therapy and treatment of autoimmune diseases.

What are SAgs doing for the bacteria?

After more than two decades of intensive research, the question of why SAgs are important for the bacteria remains largely unanswered. There are currently 11 SAgs found in *S. pyogenes* and many of them have orthologues in other streptococci, all of them sharing a common protein fold and the same target receptors on host cells, MHC class II and TcR. The evolutionary advantage of SAg production seems therefore eminent and is supported by the fact that SAg-producing streptococci only infect hosts with adaptive immunity. Furthermore, most SAgs show allelic variation, in particular SMEZ with >50 variants, that results in antigenic rather than functional differences. This confirms that SAg evolution is mainly driven by host immunity. It is almost certain that the role of SAgs is not to induce systemic lethal shock in the host. Significant antibody responses to bacterial SAgs are commonly found in healthy adults, indicating that SAg exposure must occur during either non-severe infections or asymptomatic colonization (Basma, et al., 1999). A possible advantage of SAg production might involve the corruption of the host immune response. SAgs interfere with the adaptive immune system resulting in profound Th1 type responses with non-specific T cell proliferation and massive release of type 1 cytokines, such as IL-2, IFN-γ and TNF-α. This might suppress local inflammation at the site of infection, although there is

no evidence that SAgs directly enhance colonization. By promoting a Th1 type response, SAgs might also suppress a type 2 response and prevent the production of high-affinity cytotoxic antibodies. Another possible mechanism of how SAgs corrupt the immune system might be their ability to induce T cell anergy, a non-responsive state that results from the systemic stimulation of T cells by SAgs. Anergic T cells are unable to produce IL-2 and therefore SAg stimulation might lead to local IL-2 deficiency, which could limit the expansion of antigen-specific T cells (Lavoie, Thibodeau, Erard, & Sékaly, 1999; Miller, Ragheb, & Schwartz, 1999; O'Hehir & Lamb, 1990). More recently, Llewelyn and colleagues suggested that SAgs are able to induce a regulatory T cell phenotype restricted only by the Vβ specificity of the toxin or toxins produced. They showed that stimulation of PBMCs with SPE-K (previously SPE-K/L) resulted in the rise of CD4(+) CD25(+) T regulatory T cells (Tregs) from CD4(+) CD25(-) T cells. This was Vβ-specific and required APCs. Furthermore, the Tregs expressed the anti-inflammatory cytokine IL-10 at lower SAg concentrations than was required to trigger IFN-γ production (Taylor & Llewelyn, 2010). It was later shown that SAgs are also potent inducers of human regulatory CD8(+) T cells, which were able to suppress the proliferation of CD4(+) CD25(-) T cells in response to anti-CD3 stimulation in a cell contact dependent mode. SAg induced stimulation of Tregs might therefore be a feature of acute bacterial infections contributing to immune evasion by the microbe and disease pathogenesis (Taylor, Cross, & Llewelyn, 2012). In a recent study, Kasper *et al*. have shown that acute *S. pyogenes* infection in the nasopharynx of mice is dependent upon both bacterial SAgs and host MHC class II molecules (Kasper, et al., 2014). *S. pyogenes* was rapidly cleared from the nasal cavity of wild-type C57BL/6 (B6) mice, but infection was enhanced up to ~10,000-fold in B6 mice that express the human MHC class II molecule HLA-DQ8. This infection phenotype was dependent on the production of SPE-A since an *speA*$^-$ isogenic strain showed markedly reduced infection in the noses of the B6–DQ8 transgenic mouse. Moreover, pre-vaccination with an MHC class II binding mutant toxoid of SPE-A inhibited infection. This is the first study to show that survival of *S. pyogenes* in a common niche is indeed enhanced by the production of a SAg, and gives some credence to the long held notion that SAgs are indeed virulence as well as pathogenicity factors.

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