Chemical Immunology and Allergy
Editors: J. Ring, L. Adorini, C. Berek, K. Blaser,
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Vol. 93

Superantigens and Superallergens

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Chemical Immunology and Allergy

Vol. 93

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Volume Editor

Gianni Marone, Naples

42 figures, 1 in color, and 5 tables, 2007

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Chemical Immunology and Allergy
Formerly publisched as 'Progress in Allergy' (Founded 1939),
continued 1990–2002 as 'Chemical Immunology'
Edited by Paul Kallos 1939–1988, Byron H. Waksman 1962–2002

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Bibliographic Indices. This publication is listed in bibliographic services, including Current Contents® and Index Medicus.

www.karger.com
Printed in Switzerland on acid-free paper by Reinhardt Druck, Basel
ISSN 1660–2242
ISBN 978–3–8055–8266–7

Dedication

To my late mother, *Francesca Rita Marsella*,
as a token of my gratitude for her love and support.

Contents

Preface

Superantigens constitute a growing family of bacterial and viral proteins that share the capacity of inducing massive activation of the immune system. This concept was first introduced in the late 1980s by the group of Philippa Marrack to describe the ability of staphylococcal enterotoxin B to induce a remarkable expansion of T cells expressing T cell receptors with a specific subset of the T cell receptor β-chain variable region. The classical superantigens are the T cell superantigens. However, some naturally occurring proteins possess the properties of superantigens for B lymphocytes. B cell superantigens are proteins endowed with immunoglobulin-binding capacity. Protein A of *Staphylococcus aureus* is the prototype B cell superantigen. Other B cell superantigens are gp120 of HIV-1, protein L and the human gut-associated sialoprotein known as 'protein Fv'. B cell superantigens, by interacting with membrane-bound IgE, activate human basophils and mast cells that express the high-affinity receptor for IgE. In this context, the definition of immunoglobulin superantigens has been transferred to superallergens.

In this volume, we have tried to cover novel aspects of T cell and B cell superantigens and some recent molecular and clinical findings generated by the superallergen concept. In particular, the recent completion of several genome projects and database mining led to the identification of a myriad of novel superantigens. We also focused on the possibility that certain superantigens can modulate regulatory T cells and that superantigens may stimulate or inhibit IgE synthesis depending on the conditions.

An important part of this volume is devoted to the significance of superantigens in a wide spectrum of clinical settings going well beyond the classical

superantigen-associated diseases. For instance, there is some evidence that superantigens/superallergens might play a role in certain aspects of HIV-1 infection and autoimmune diseases. In addition, there is growing evidence that staphylococcal superantigens and superallergens might be involved in certain diseases of the upper and lower respiratory tract. For instance, endogenous, viral and bacterial superallergens can activate primary effector cells of allergic reactions to release proinflammatory mediators and cytokines.

The rapid advances in this field make it difficult to produce a timely reference text. Despite this difficulty, I accepted the invitation of the Editors of the *Chemical Immunology and Allergy* series to produce a volume entitled *Superantigens and Superallergens.* This project was designed to highlight results obtained with the recent characterization of molecular and clinical aspects of T and B cell superantigens and of superallergens. Several issues remain to be solved. The evolution of pathogens and their hosts is inextricably intertwined and studies of superantigens/superallergens have revealed interesting dimensions in the complex ongoing battle between pathogens and their hosts. We should also remember that we are constantly exposed to superantigens and superallergens. This leads to the question: 'why do bacteria and viruses produce superantigens?' All these fascinating aspects are awaiting answers.

There are several important aspects still remaining to be fully addressed. First, novel bacterial and viral superantigens and superallergens should be identified. Secondly, we need to know more about the molecular events governing the immunological synapse induced by superantigens and certain superallergens. Similarly, there is still much to learn about the activation of different subsets of T and B cells, and of effector cells caused by superantigens and superallergens. The information that might arise from such studies may lead to the prevention and better management of superantigen/superallergen-associated diseases.

It has been a rewarding experience for me to interact with many friends and colleagues and I am pleased to acknowledge the excellence of their work. I would like to thank Karger publishers and their staff, as well as Jean Gilder for their assistance throughout the production of this volume.

This volume owes much to the stimulating intellectual environment provided by my colleagues at the Center for Research in Basic and Clinical Immunology (CISI) of the University of Naples Federico II.

Gianni Marone

Marone G (ed): Superantigens and Superallergens.
Chem Immunol Allergy. Basel, Karger, 2007, vol 93, pp 1–23

Streptococcal Superantigens

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Abstract

Superantigens (SAgs) are the most powerful T cell mitogens ever discovered. They activate the immune system by binding to the major histocompatibility complex (MHC) class II and T cell receptor molecules. One of the major producers of SAgs is *Streptococcus pyogenes*, or group A streptococcus (GAS). The recent completion of several GAS genome projects resulted in a sharp rise of novel streptococcal SAgs that were identified by database mining. Orthologue genes of several streptococcal SAgs have also been found in non-GAS, such as *Streptococcus equi* and *Streptococcus disgalactiae.* Crystal structure analyses have shown a common protein fold for all streptococcal SAgs analyzed thus far. Furthermore, co-crystal structures of SAgs complexed with MHC class II and T cell receptor β-chains, respectively, have provided further insight into the molecular interactions of these toxins with their host cell receptors. This chapter will also discuss the potential involvement of SAgs in severe GAS disease, in particular the highly lethal streptococcal toxic shock syndrome.

Streptococcus pyogenes, or Lancefield group A streptococcus (GAS), is a Gram-positive commensal bacterium that colonizes human tissue. It is estimated that between 5–15% of healthy individuals harbor the bacterium, usually in the respiratory tract, without causing disease. However, when the bacteria are able to penetrate the physical barrier of the skin (e.g. wound infection) or when the host immune system is compromised, *S. pyogenes* can cause a wide range of diseases. Mild GAS diseases include pharyngitis and tonsillitis, which occur primarily among children 5–15 years of age and in confined institutions, like military camps. If untreated, these diseases can develop into severe conditions such as rheumatic heart disease, acute rheumatic fever (ARF), and poststreptococcal acute glomerulonephritis [1, 2]. Penetration of the skin barrier by GAS can cause bacteremia and severe invasive deep tissue infections, such as cellulitis, myositis, and necrotizing fasciitis (also known as the ‘flesh-eating disease’)

[3–6]. Since the 1980s, there has been an alarming increase in GAS-mediated invasive disease, not only in frequency, but also in severity. The severest form of invasive infection results in clinical symptoms similar to staphylococcal toxic shock syndrome and was therefore described as streptococcal toxic shock syndrome (STSS). Characteristic symptoms of STSS include hypotension, fever, rash, vomiting, diarrhea, multiple organ failure, and shock [7].

GAS produce a wide range of virulence factors, including several adhesion molecules and a large number of secreted proteins (>30) comprising the streptococcal superantigens (SAgs), cytolysins, proteases, DNases, and various hydrolytic enzymes. The streptococcal SAgs are monomeric proteins, expressed as precursor molecules with typical bacterial signal peptides. The secreted mature toxins have molecular weights of 23.6–27.4 kDa (table 1).

The term 'superantigen' was coined in 1989 in the research group of Philippa Marrack and John Kappler and describes a group of proteins with extremely high potency to stimulate human, and to a certain degree, other mammalian CD4 and CD8 T cells [8]. In contrast to conventional peptide antigens, SAgs bind to the major histocompatibility complex (MHC) class II outside the peptide-binding groove, and sequentially to the T cell receptor (TCR) via the variable region of the TCR β-chain [9–11]. Since the number of different Vβ regions in the human T cell repertoire is restricted to less than 50, comprising about 24 major types of Vβ elements, and since most SAgs can bind more than one Vβ, up to 25% of an individual's T cell population can be activated [10, 12]. This is in sharp contrast to the 1 in 10^5-10^6 naïve T cells (0.001–0.0001%) that are responsive to conventional peptide antigen. Consequently, each SAg is associated with a characteristic Vβ signature that is independent of the MHC polymorphism (table 1). The oligoclonal stimulation of T cells and antigen-presenting cells by SAgs results in a massive release of proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β), and T cell mediators, such as IL-2, which can lead to fever and shock [10, 13–17]. Concentrations of less than 0.1 pg/ml of a bacterial SAg are sufficient to stimulate T cells in an uncontrolled manner, making SAgs the most powerful T cell mitogens ever discovered [18]. The half-maximal response to streptococcal SAgs (P_{50} value), which describes the concentration of toxin needed for a 50% stimulation of T cells, is between 0.02–50 pg/ml for human T cells (table 1).

Group A Streptococcal Superantigens

The discovery of streptococcal SAgs can be divided into two major periods: the pregenomic era and the postgenomic era. During the pregenomic

Table 1. Functional properties of streptococcal superantigens

SAg	MW (kDa)	Organism	Crystal structure solved	Zinc binding	MHC II binding α/β chain	Human TCR Vβ specificity	P_{50} (h) (pg/ml)	References
SPE-A	26.0	*S. pyogenes*	+	+	+/−	2.1, 12.2, 14.1, 15.1	?	[19, 23, 24, 25, 56]
SPE-C	24.4	*S. pyogenes*	+	+	−/+	2.1, 3.2, 12.5, 15.1	0.1	[21, 31, 32, 57, 65]
SPE-G	24.6	*S. pyogenes*	−	+	−/+	2.1, 4.1, 6.9, 9.1, 12.3	2	[36]
SPE-H	23.6	*S. pyogenes*	+	+	−/+	2.1, 7.3, 9.1, 23.1	50	[36, 58]
SPE-I	26.0	*S. pyogenes*	−	+	−/+	6.9, 9.1, 18.1, 22	0.1	[39]
SPE-J	24.6	*S. pyogenes*	+	+	−/+	2.1	0.1	[39, 40, 59]
SPE-K/L	27.4	*S. pyogenes*	−	+	−/+	1.1, 5.1, 23.1	1	[41, 42, 43]
SPE-L/M	26.2	*S. pyogenes*	−	+	−/+	1.1, 5.1, 23.1	10	[43, 44]
SPE-M	25.3	*S. pyogenes*	−	+	?	1.1, 5.1, 23.1	?	[44]
SSA	26.9	*S. pyogenes*	−	−	+/−	1.1, 3, 15	?	[33]
SMEZ-1	24.3	*S. pyogenes*	−	+	−/+	2.1, 4.1, 7.3, 8.1	0.08	[34, 35]
SMEZ-2	24.1	*S. pyogenes*	+	+	−/+	4.1, 8.1	0.02	[36, 37, 58]
SePE-H	23.6	*S. equi*	−	+	−/+	?	?	[46]
SePE-I	25.7	*S. equi*	−	+	−/+	?	?	[46]
SPE-L_{Se}	27.4	*S. equi*	−	+	−/+	?	?	[43]
SPE-M_{Se}	26.2	*S. equi*	−	+	−/+	?	?	[43]
SPE-G^{dys}	24.4	*S. dysgalactiae*	−	+	−/+	?	?	[48]
SDM	25.0	*S. dysgalactiae*	−	+	−/+	1.1, 23	?	[47]

List of all currently known streptococcal SAgs. The major T cell receptor Vβ targets are underlined.
P_{50} (h) = Concentration needed for half maximum proliferation of human T cells.

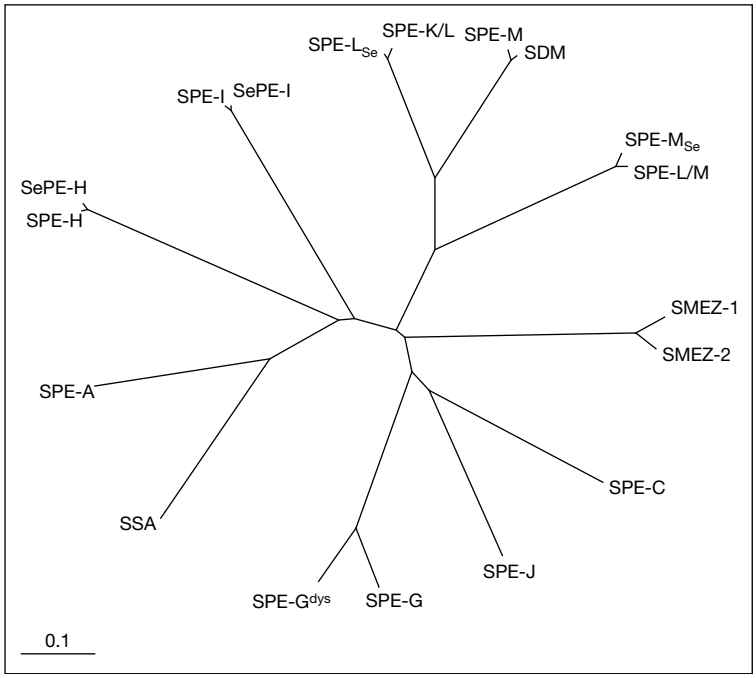

Fig. 1. Streptococcal SAg family tree. The tree was created using Clustal W and TreeViewPPC version 1.5.3 and is based on primary amino acid sequence homology of the mature toxins.

era, streptococcal SAgs were identified in cell culture supernatant based on their mitogenic activity. The toxins were biochemically enriched to a purity that was sufficient for functional studies and for analysis by N-terminal sequencing. In the postgenomic era, novel streptococcal SAgs were identified by database mining of GAS genomes and recombinant forms of the toxins were used for functional studies. Eleven GAS SAgs are currently known, but the number will most likely increase when more GAS genomes are analyzed. The GAS SAgs share amino acid sequence identities between 17 and 48% (fig. 1). Furthermore, the streptococcal SAgs are part of an even larger family together with the structurally related staphylococcal SAgs and the staphylococcal SAg-like proteins.

The Pregenomic GAS SAgs

In 1924, Dick and Dick [19] observed scarlet fever-like symptoms in healthy volunteers after intradermically injecting them with culture filtrates

from hemolytic streptococci of scarlatinal origin. They postulated that this reaction (also known as the 'Dick reaction') was caused by an erythrogenic toxin, which they named 'scarlet fever toxin'.

A second toxin (toxin B) was identified in 1934 [20] and yet another toxin was isolated from culture filtrate of a serotype M18 strain, associated with scarlet fever and named toxin C [21].

Since pyrogenicity (the ability to induce fever) was believed to be the primary effect of these toxins, Kim and Watson [22] designated the toxins as streptococcal pyrogenic exotoxins A, B and C (SPE-A, SPE-B, SPE-C). In the 1980s, the *speA* gene was identified and cloned from bacteriophage T12. DNA analysis revealed an open reading frame consisting of 756 base pairs encoding a 252-amino-acid precursor protein [23]. The N-terminal 30 amino acids represent a signal peptide that is removed during secretion to produce the mature toxin. Recombinant SPE-A was produced in *Bacillus subtilis* and shown to mimic the biological activity of native preparations [24]. In 1990, it was demonstrated that SPE-A activates murine T cells in an MHC-II-dependent and TCR Vβ-specific mode, finally establishing its function as an SAgs [25].

The *speB* gene was cloned from a serotype M12 GAS strain [26] and shown to be identical to streptococcal cysteine protease confirming earlier results that showed immunological cross-reactivity between SPE-B and streptococcal cysteine protease [27]. Earlier reports that demonstrated SPE-B-induced T cell proliferation [28] were later disputed by others, who used SPE-B preparations of very high purity and argued that the initial findings were due to contamination with an unknown SAg [29, 30]. It is now widely accepted that SPE-B is not an SAg.

The *speC* gene was cloned from the chromosome of *S. pyogenes* strain T18P by Schlievert and colleagues [31]. The status of SPE-C as an SAg was established in 1991, when Leonard et al. [32] demonstrated MHC II/TCR Vβ-dependent T cell mitogenicity.

Another novel SAg was discovered in the cell culture supernatant of *S. pyogenes* strain Weller (serotype M3) and named streptococcal superantigen (SSA) [33]. The *ssa* gene showed a higher degree of DNA sequence similarity to the staphylococcal enterotoxins B, C_1 and C_3 (SEB, SEC_1 and SEC_3), than to SPE-A or SPE-C. SSA targets T cells bearing the Vβ1, 3 and 15 regions, in contrast to SPE-A, which mainly targets Vβ12 and 14, or SPE-C, which mainly targets Vβ2 (table 1).

Yet another novel SAg was reported by Kamezawa et al. [34] and named streptococcal mitogenic exotoxin Z (SMEZ). This toxin was purified from an *S. pyogenes* serotype M1/T1 strain and showed a unique TCR binding specificity targeting mainly Vβ4 and Vβ8 regions. An allelic variant of this toxin

was later purified from an *S. pyogenes* serotype T12 strain and designated SPE-X/SMEZ3 [35].

DNA sequence analysis of a *smez* gene cloned from *S. pyogenes* 2035 showed nucleotide changes in 36 positions (= 5%) compared to the *smez* gene and was designated *smez-2* [36]. The deduced protein sequence differs in 17 amino acid residues (= 8.1%) and the most significant difference is an exchanged pentapeptide sequence at position 96–100, where the EEPMS sequence of SMEZ is converted to KTSIP in SMEZ-2. In a follow-up study, *smez* DNA sequences of 37 GAS isolates were analyzed, representing 22 different M/*emm* types, 5 M-nontypeable isolates and 2 isolates of unknown M-type [37]. As a result, 22 novel *smez* alleles were identified, which appear to be in linkage equilibrium with the M/*emm* type. Three of the *smez* sequences (*smez-6, smez-19* and *smez-23*) represent pseudogenes that have single base pair deletions causing frame shifts. Interestingly, the *smez* gene showed significant mosaic structure suggesting that the polymorphism has arisen from homologous recombination events rather than random point mutations. The biological function of SMEZ, e.g. binding to MHC class II and Vβ-specific stimulation of T cells, was not affected by the allelic variation. However, the SMEZ variants showed antigenic differences. Neutralization responses of individual human sera from healthy donors against different SMEZ variants varied significantly [37].

The Postgenomic GAS SAgs

The completion of the first *S. pyogenes* sequencing project (a serotype M1 strain) was the start in microbial genomics of GAS [38]. The University of Oklahoma *S. pyogenes* M1 database was accessible on the Internet from the very beginning of the project and contained regularly updated sequence contigs derived from a shotgun plasmid library. The DNA sequence database could be mined for genes of interest from anywhere in the world long before completion of the project. Although streptococcal and staphylococcal SAgs only share limited DNA sequence homology (sometimes less than 25%), they all share the two 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(3)-R-X(2)-L-X(5)-[LIV]-Y (PS00278). Mining of the *S. pyogenes* M1 genome database with these two motifs resulted in the discovery of four novel *sag* genes, which were designated *speG, speH, speI* and *speJ* [36, 39]. The genes were cloned and expressed in *Escherichia coli*. Functional analysis using recombinant forms of the novel toxins showed that they bound to MHC II and stimulated T cells with high potencies in a Vβ-dependent mode, confirming their predicted role as SAgs. Furthermore, SPE-J induced fever in rabbits and was lethal in two rabbit

models of STSS [40]. In addition, a putative gene fragment encoding a non-functional peptide with sequence similarity to the C-terminal region of SPE-C was found and named *speK* [38].

Another novel *sag* gene, *speL*, was identified in Japan after DNA analysis of the 41,769-bp temperate phage ΦNIH1.1 from a serotype M3 strain [41]. In the same year, a complete *S. pyogenes* M3 genome was analyzed in the USA and led to the discovery of the same *sag* gene, which was designated *speK* [42], a name that had previously been assigned to a pseudogene on the *S. pyogenes* M1 genome [38]. In this chapter, we will refer to this SAg as SPE-K/L. The *speK/L* gene was also found on a serotype M89 strain from New Zealand and named *speL* in accordance with the Japanese designation [43]. It was identified by PCR analysis with a specific DNA primer pair designed from a novel *sag* gene that was discovered by mining of a *Streptococcus equi* database at the Sanger Center and named $speL_{Se}$ (see below). The same strategy also led to the discovery of a hitherto unknown *sag* gene in serotype M80 that was designated *speM* [43]. The SAg nomenclature turned increasingly confusing, when another *S. pyogenes* genome (serotype M18) was completed in the USA revealing two novel *sag* genes, which were named *speL* and *speM* [44]. *speL* is identical to *speM* found on the M80 strain and will here be referred to as *speL/M,* while *speM* from the M18 strain had not been reported before. Recombinant forms of SPE-K/L and SPE-L/M were shown to be potent mitogens targeting T cells in a Vβ-specific and MHC II-dependent mode, which confirmed their role as SAgs [43].

Superantigens of Group C and G Streptococci

Group C and G streptococci (GCS and GGS) are commonly regarded as commensals and usually found in association with the normal flora of human skin, the pharynx and intestine. Recently, there have been an increasing number of reports implicating GCS and GGS with severe invasive infections, such as necrotizing fasciitis and toxic shock syndrome [45]. Although mitogenic activity in supernatant of clinical GCS and GGS isolates had been reported over several years, SAgs had not been identified until recently. The first report came from Timoney's group who identified two SAgs in *S. equi*, a bacterium that causes strangles in horses, but can also infect humans [46]. The *S. equi* pyrogenic exotoxins (SePE) H and I are highly homologous to their *S. pyogenes* counterparts SPE-H and SPE-I (>98% amino acid sequence identities), indicating another horizontal gene transfer from *S. pyogenes* to *S. equi* or vice versa. Interestingly, both genes, *sepeH* and *sepeI*, were not found in the closely related *Streptococcus zooepidemicus* and it was suggested that the acquisition of the *sag* genes might be an important event in the formation of a more virulent

S. equi strain from its putative *S. zooepidemicus* ancestor [46]. Another two *sag* genes were identified by data mining of the *S. equi* genome at the Sanger Center and named $speL_{Se}$ and $speM_{Se}$, due to the homology to their *S. pyogenes* counterparts *speL* and *speM* (here described as *speK/L* and *speL/M*) with 99 and 98.1% nucleotide identities, respectively [43].

Two SAgs have been identified from *Streptococcus dysgalactiae* subsp. *equisimilis* called *S. dysgalactiae*-derived mitogen (SDM) [47] and SPE-G^{dys} [48]. SDM is 99% similar to SPE-M and SPE-G^{dys} is 86% similar to SPE-G from *S. pyogenes*. Amino acid exchanges are outside the MHC class II and TCR binding sites suggesting that the GAS toxins and the non-GAS toxins are orthologues with identical functions.

A recent study has shown that GAS, GCS and GGS exchange housekeeping genes by horizontal gene transfer with strong net directionality of gene movement from GAS donors to GCS and GGS recipients [49]. The fact that all GAS counterparts of GCS/GGS *sag* genes, except *speG*, were found on prophages and the strong homology between the orthologues suggests that commensal GCS/GGS strains might have acquired these *sag* genes from pathogen GAS generating more virulent GCS and GGS.

Regulation of Superantigen Gene Expression

SAgs are generally expressed at very low concentrations from strains grown in vitro. It is widely believed that SAg expression is upregulated when the bacteria encounter their host and that host factors might play a role in *sag* gene regulation. Evidence was first provided by a study using a mouse infection chamber model that allows sampling of *S. pyogenes* isolates at various time points after infection [50]. In this study, micropore Teflon diffusion chambers were implanted subcutaneously into BALB/c mice and inoculated with an *S. pyogenes* strain that produced only minor amounts of SPE-A when grown in vitro. A significant increase in SPE-A expression was detected in the infected mice 7 days after infection. SPE-A expression was still high after 21 in vitro passages of the isolate suggesting a stable switch of the *speA* gene. In another study, *S. pyogenes* was co-cultured with human pharyngeal cells resulting in the induction of lysogenic phage as well as the phage-associated SPE-C [51]. The authors also demonstrated that the induction was caused by a small (1–10 kDa) heat- and protease-resistant molecule produced by the pharyngeal cell line.

Co-culture of strain MGAS315 with Detroit 562 human epithelial pharyngeal cells induced prophage Φ315.4, which encodes *speK/L*. However, no significant production of SPE-K/L was observed [52].

The recent completion of several GAS genome projects combined with DNA microarray-based transcription analysis provided further evidence for the importance of host factors in the regulation of GAS virulence genes, including SAgs. A transcriptome study with a serotype M1 strain grown in human blood showed upregulation of *speA, speG, speJ* and *smez* [53]. Notably, *speG, speJ* and *smez* were upregulated after 30 min (62-, 264- and 52-fold, respectively), but expression was reduced after 60 min. In contrast, *speA* was upregulated continuously, showing a 483-fold increase after 30 min and a 2,139-fold increase of mRNA after 90 min. The transcriptome of a serotype M1 strain was also analyzed during an 86-day infection protocol in 20 cynomolgus macaques and revealed differences in *sag* expression in distinct disease phases [54]. Expression of *speA, speJ* and *smez* increased during the colonization phase. However, increased *speJ* expression started at the early stage of infection and could be correlated to low GAS cell densities. In contrast, *speA* and *smez* were upregulated at a later stage, when GAS cell densities increased. Interestingly, *smez* expression could be correlated with peak levels of C-reactive protein, a sensitive indicator of inflammation. Furthermore, *smez* was the most dominant acute-phase-correlated proinflammatory gene, but expression did not correlate with peak increases of pharyngitis or tonsillitis.

The molecular mechanisms of streptococcal *sag* gene regulation are largely unknown. However, a study has shown that the *speA* gene and several other virulence genes are under the control of the Nra transcription regulator, a member of the RofA-like protein family [55]. Nra represses the expression of several virulence genes, including *speA*. Maximum expression of Nra occurs in the early stationary growth phase, but does not appear to respond to changing atmospheric conditions.

Protein Structure of Superantigens

The crystal structure of five streptococcal SAgs, SPE-A [56], SPE-C [57], SPE-H [58], SMEZ-2 [58] and SPE-J [59], has been solved. All SAg protein structures show a conserved two-domain architecture and the presence of a long, solvent-accessible α-helix spanning the center of the molecule (fig. 2a).

The N-terminal domain is a five-stranded mixed β-barrel with Greek key topology. This structure belongs to a larger 'structural classification of proteins' known as the oligosaccharide/oligonucleotide-binding fold (OB fold), which currently consists of 8 superfamilies. One of them, the 'bacterial enterotoxin' superfamily, comprises the 'SAg toxin N-terminal domain' family and the 'bacterial AB_5 toxin' family [60]. The 'SAg toxin N-terminal domain' family includes the staphylococcal SAgs and the recently discovered staphylococcal

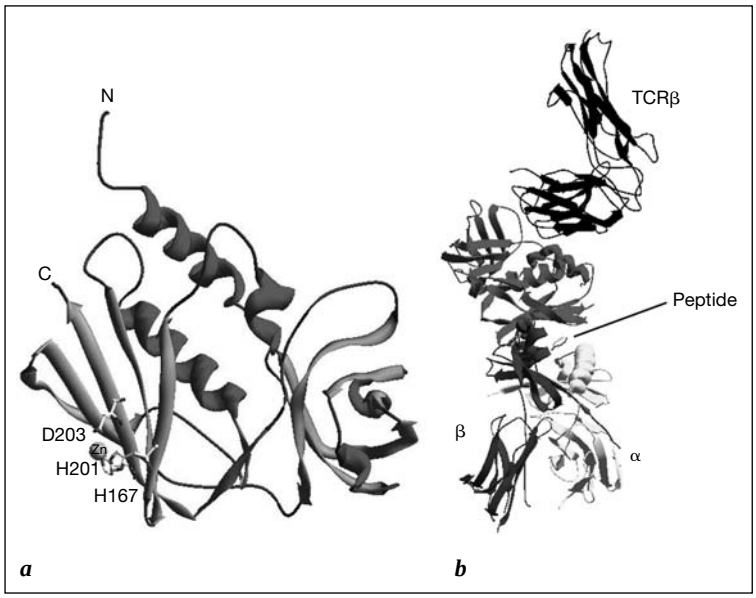

Fig. 2. ***a*** Crystal structure of SPE-J [59]. All streptococcal SAgs share a common protein fold consisting of an N-terminal mixed β-barrel domain (right) and a C-terminal β-grasp motif (left) separated by a central solvent-accessible α-helix. The 'high affinity' MHC class II β-chain zinc-binding site is located within the C-terminal domain as indicated. ***b*** Model of the trimolecular MHC class II-SAg-TCR Vβ complex. The model was created by superposition of the SPE-C/HLA-DR2a structure (Brookhaven database entry 1HQR) with the SPE-C/hTCR Vβ2.1 structure (1KTK).

SAg-like toxins (SSLs), which lack SAg activity. It has been suggested that all members of this superfamily derived from a common ancestor by divergent evolution and express their toxicity by using the OB fold binding face to bind cell surface proteins or oligosaccharides [60].

The C-terminal domain comprises a four-stranded β-sheet capped by the central α-helix and is structurally related to the β-grasp motif, which is also present in other proteins, such as ubiquitin and immunoglobulins.

Molecular Interactions of Superantigens with Receptor Molecules

MHC Class II Binding

All SAgs bind to MHC class II as a prerequisite for T cell recognition. The molecular mechanism of this interaction is not only different from the binding

of conventional peptide antigens, but also significantly varies between individual SAgs. In general, SAgs bind to the invariant MHC class II α-chain or to the polymorphic MHC class II β-chain. The staphylococcal SAgs toxic shock syndrome toxin-1 (TSST-1) and SEB are the prototype molecules for MHC class II α-chain binding. Structural studies of SAgs complexed with HLA-DR have shown that these toxins possess an exposed hydrophobic loop region within the N-terminal β-barrel domain to bind to a hydrophobic groove located in the distal region of the DR α_1-domain with binding affinities of 10^{-5} M [61, 62]. This interaction has been referred to as the 'generic' or the 'low-affinity' binding site. Only two streptococcal SAgs, SPE-A and SSA, bind to the MHC class II α-chain. The overall structure of SPE-A is more closely related to SEB and SEC than to any other streptococcal SAg [56] and it has been shown that SPE-A competes with SEB for binding to HLA-DR1 molecules, suggesting that both toxins have a common recognition site for MHC class II [63]. However, there are differences in binding affinity for MHC class II. SPE-A has a greater affinity for HLA-DQ than it does for HLA-DR or HLA-DP, while SEB has a greater affinity for HLA-DR, suggesting that SPE-A and SEB have similar but not identical MHC class II binding sites.

Apart from SPE-A and SSA, all other streptococcal SAgs appear to bind to MHC class II β-chain. The first evidence for this mode of binding was provided for SEA, when Herman et al. [10] showed that SEA binds effectively to most HLA-DR alleles and isotypes, such as DR1, but only poorly to DRw53. The difference in binding was localized to a single amino acid residue at position 81 in the β_1-domain, a highly conserved histidine that is replaced by a tyrosine in DRw53. It was later shown that His81 is part of a zinc coordination that also involves SEA residues His187, His225 and Asp227 [64]. The relative binding affinity of this interaction is about 100-times higher than the generic low-affinity site (10^{-7} M) and has therefore been referred to as the 'high-affinity binding site'. The first streptococcal SAg shown to possess this mode of binding was SPE-C. Li et al. [65] reported that SPE-C binding to MHC class II was completely abolished in the presence of EDTA, but could be restored by excess of Zn^{2+} over EDTA. Moreover, SPE-C did not compete with SEB and TSST for binding to MHC class II. Structural analysis of SPE-C revealed a zinc-binding site in the C-terminal domain, similar to that in SEA involving SPE-C residues His167, His201, and Asp203 [57]. The complete zinc coordinated binding was finally confirmed with the successful co-crystallization of SPE-C with HLA-DR2a bearing a peptide derived from myelin basic protein. The structure not only showed the conserved tetrameric zinc complex, but also revealed extensive interaction of the bound peptide [66] (fig. 2b). Crystal structures and computer-generated models of SPE-G, SPE-H, SPE-I, SPE-J, SPE-K/L, SPE-L/M, SPE-M and SMEZ showed the conserved zinc-binding

site in the C-terminal domain of all of these proteins. Zinc dependency was demonstrated by biochemical analysis, when binding to MHC class II was completely abolished in the presence of EDTA, but could be restored by excess of Zn^{2+} over EDTA [36, 39]. Furthermore, these toxins lack the generic MHC class II α-chain binding motif and did not prevent SEB and TSST from binding to MHC class II in competition experiments [36, 39, 43]. On the other hand, Scatchard plot analysis of SPE-G, SPE-H, SMEZ-1 and SMEZ-2 revealed two distinct binding affinities: a high-affinity binding of 16–65 nM, similar to the zinc-dependent MHC class II β-chain binding of SEA, and a low-affinity binding of 1–2 μM. Moreover, these toxins only partially competed against each other for MHC class II binding with a clear hierarchical order [(SPE-C, SMEZ-1) > SMEZ-2 > SPE-H > SPE-G], where SPE-G did not compete with any other toxin. Due to the lack of any potential MHC class II α-chain binding motifs in the toxin sequences, it was suggested that some toxins have a more restricted repertoire of MHC class II molecules defined by the bound antigenic peptide and that the multiphasic Scatchard plots resulted from a variety of affinities ranging from nanomolar to micromolar defined by the bound peptide [36]. Further evidence was provided when the SPE-C-HLA-DR2a structure revealed extensive interaction of the bound peptide [66]. This raises the possibility that certain bound peptides could enhance the potency of the SAg by promoting high-affinity, but low-density binding to MHC class II [67, 68]. This mechanism might be important to avoid T cell apoptosis through supraoptimal signalling caused by high ligand densities.

SPE-C was shown to form homodimers using its secondary zinc-binding site. The dimer interface is located opposite from the high-affinity HLA-DR binding site at a position that corresponds to the generic binding site. Consequently, SPE-C interacts with MHC class II molecules forming DRβ-SPE-C-SPE-C-DRβ complexes [57, 65]. The biological function of SPE-C dimerization is unknown, but it has been shown that cross-linking of MHC class II upregulates the expression of costimulatory molecules, such as B7, and cell adhesion molecules on antigen-presenting cells and is required for inflammatory cytokine expression [69]. The recently solved crystal structure of SPE-J also revealed a dimer structure [59]. Interestingly, the formation of an SPE-J dimer would prevent the molecule from binding to the TCR suggesting an alternative function of these SAgs, apart from T cell activation. A similar mechanism has recently been demonstrated for SSA [70]. Monomeric and dimeric forms of SSA were found in supernatants of GAS cultures. Dimer formation occurred via an intermolecular cysteine bridge involving Cys26 and, like in the SPE-J dimer, most probably occludes the TCR interaction site.

T Cell Receptor Binding

The first two co-crystal structures of a streptococcal SAg complexed with a TCR β-chain (SPE-A binding to murine TCR Vβ8.2 and SPE-C binding to human TCR Vβ2.1) were published in 2002 by Mariuzza and colleagues [71] (fig. 2b). The SPE-A-mVβ8.2 complex is quite similar to the previously solved SEB-mVβ8.2 structure [72], which is probably not much of a surprise considering the structural homology between SPE-A and SEB. The TCR binding site is located in a shallow groove between the two globular domains of the SEB/SPE-A molecules created by residues from the α_2-helix, the β_2-β_3 loop, the β_4-strand, the β_4-β_5 loop, the β_5-strand and the α_5-helix. In the mTCR Vβ8.2 molecule, residues from the complementarity-determining region 2 (CDR2), framework region 2 (FR2) and, to a lesser extent, hypervariable region 4 and FR3 were found to be involved in SAg recognition. However, there are two significant structural differences between the two complexes. In addition to intermolecular contacts with CDR2, FR3 and hypervariable region 4, SPE-A also interacts with the mVβ8.2 CDR1 loop via a hydrogen bond between Glu94 on the disulfide loop region of SPE-A and Asn28 on the mVβ8.2 CDR1 loop. In contrast, the positionally equivalent residue in SEB (Thr99) is unable to form an intermolecular contact with the CDR1 loop due to a difference in side chain length. There are also a number of other hydrogen bonds that involve side chain atoms of both, SPE-A and mVβ8.2, which is in sharp contrast to the exclusive use of main chain contacts in the SEB-mVβ8.2 complex.

The interaction between SPE-C and hVβ2.1 is quite different and includes a significantly larger buried surface area than the other complexes. The number of intermolecular contacts is higher and also includes the highly variable hVβ2.1 CDR3 loop. Interestingly, SPE-C displays a much higher specificity to stimulate T cells than SEB or SPE-A targeting primarily the hVβ2.1 TCR. It has been proposed that this specificity derives from two single amino acid insertions, one each in CDR1 and CDR2, and an extended CDR3 loop. The CDR1 insertion shifts this loop towards the SPE-C molecule, resulting in additional intermolecular contacts, while the CDR2 insertion produces a noncanonical CDR2 loop that positions the inserted residue for optimal contact with SPE-C.

Based on these observations, three categories of SAg-TCR complexes have been proposed: (a) highly promiscuous T cell activators, including SEB and SEC_3, that bind TCR β-chains in a simple conformation-dependent manner and interact only with a single CDR loop, CDR2; (b) moderately promiscuous T cell activators, including SPE-A, that form direct side chain/side chain contacts overlaid onto the conformation dependence of the first group, and the additional involvement of the CDR1 loop; (c) highly selective T cell activators,

including SPE-C, that bind to TCR Vβ domains with the highest degree of structural dissimilarity, and the usage of all three CDR loops [73].

Streptococcal Superantigens and Human Disease

Invasive GAS Disease and STSS

GAS is associated with a number of severe invasive diseases, including cellulitis, myositis, and necrotizing fasciitis (or flesh-eating disease) [3–6]. The severest form of invasive GAS disease is STSS with mortality rates of 30–70%. The clinical symptoms are very similar to those in staphylococcal toxic shock, but STSS is often associated with bacteremia, myositis, or necrotizing fasciitis. STSS is characterized by rash, hypotension, and multiple organ failure. Several lines of epidemiological, experimental, and clinical observations strongly imply that at least some SAgs play a pivotal role in the pathogenesis of STSS and possibly in other invasive GAS diseases.

Epidemiological Studies

Serotype M1 and M3 strains have been predominantly isolated from patients with STSS. Both strains frequently produce SPE-A and SPE-C [74]. The *speA* gene was found in a majority (40–90%) of GAS isolates from the USA associated with invasive GAS disease and STSS, but only in a minority (15–20%) of isolates from noninvasive diseases like pharyngitis [74, 75]. A high frequency of *speA* (80%) was also observed in isolates from STSS patients collected in Australia [76]. A study by Reichardt et al. [77] of 53 strains from patients with STSS from Europe and Chile found *speA* and *speC* in 64 and 28%, respectively. In contrast, a more recent study by Hsueh et al. [78] showed no significant difference in the frequency of *speA* between invasive and noninvasive GAS isolates collected in Taiwan (39 and 36%, respectively). Furthermore, among the invasive strains, *speA* was found in only 18% of the isolates associated with STSS cases. The recent discovery of several novel streptococcal *sag* genes, with *speG* and *smez* present on all strains analyzed thus far, might explain the development of STSS in these cases. In addition, regulation of *sag* gene expression appears to play an important role (see above).

Animal Infection Models

Stevens et al. [79] showed that intravenous infusion of a SPE-A-producing serotype M3 strain resulted in profound hypotension, leukopenia, metabolic acidosis, renal impairment, thrombocytopenia and disseminated coagulopathy within 3 h in a baboon model of GAS bacteremia that mimics human STSS.

Serum TNF-α peaked at 3 h and anti-TNF-α antibodies markedly improved arterial pressure and survival, indicating the important role of TNF-α in STSS. Experiments with isogenic GAS strains lacking the *speA* gene administered into rabbits showed that only the wild-type strain caused STSS, while the *speA* knockout strain did not [80]. Sriskandan et al. [81] used an isogenic *speA–* strain to evaluate the role of SPE-A in a murine model of bacteremia and streptococcal muscle infection. Surprisingly, disruption of *speA* was not associated with attenuation of virulence and paradoxically the *speA–* mutant led to increased bacteremia and a reduction of neutrophils at the site of primary muscle infection. A possible explanation for this unexpected result is that SAgs are significantly less active on murine peripheral blood lymphocytes [36] probably due to inefficient binding to murine MHC class II. Indeed, different results were obtained in studies with HLA-DQ transgenic mice [82, 83]. Expression of HLA-DQ rendered the mice susceptible to SPE-A-induced lethal shock that was accompanied by massive cytokine production. Immune activation during GAS infection was manifested by Vβ-specific T cell repertoire changes and widespread lymphoblastic tissue infiltration. In contrast, lymphoid activation was undetectable in transgenic mice infected with an isogenic *speA–* strain, demonstrating the pivotal role of a single SAg in the pathogenesis of invasive GAS disease. In vivo experiments using an intraperitoneal model of infection demonstrated that SMEZ did not contribute to mortality or impede bacterial clearance in HLA-DQ transgenic mice, but led to a rise of Vβ11 T cells in the spleen [84]. Interestingly, infection with an isogenic M89 *smez–* strain failed to elicit a significant cytokine production compared with the parent strain, but resulted in a clear rise in murine Vβ4 T cells, suggesting a role for SMEZ as a repressor of cognate antistreptococcal response.

Clinical Studies

SPE-A has been detected by ELISA in the sera of STSS patients, and its presence was associated with elevated levels of TNF-α, providing some evidence of SPE-A-induced T cell activation [85]. In a more recent study, strong mitogenic activities were observed in the serum of two patients with STSS, one of whom died. Although the infecting GAS strains carried several *sag* genes, including *speA, speC, speG, speJ*, and *smez*, the mitogenic activity could be wholly attributed to SMEZ, with a small contribution of SPE-J in one case [86]. The quantity of circulating toxin was estimated to be in the order of 100 pg/ml. The surviving patient developed neutralizing antibodies against SMEZ during convalescence, providing further evidence for the importance of SMEZ in STSS. Furthermore, the results suggested that the lack of neutralizing antibodies against SAgs might be a risk factor for invasive GAS disease. Supportive evidence also came from other studies. Eriksson et al. [87] reported that

neutralization of SPE-A lymphocyte mitogenicity was totally absent in sera from patients with STSS and low in sera from patients with uncomplicated bacteremia, compared with levels in sera from uncomplicated erysipelas. Another study showed that the levels of neutralizing anti-SPE-A antibodies in plasma samples from severe and nonsevere invasive GAS infections were significantly lower than in age- and geographically matched healthy controls [88]. In a recent case study, a novel *smez* allele (*smez-34*) was identified in an *emm118* strain isolated from a patient with STSS. SMEZ-34 is closely related to the highly potent SMEZ-2 variant. When grown in vitro, the *emm118* isolate produced SMEZ as the major mitogenic toxin. The acute serum completely lacked protective antibodies against SMEZ-2, but the patient seroconverted during convalescence [89].

Genetic Background of the Host

Recent results suggested that the genetic background might influence the susceptibility to invasive GAS disease. In particular, HLA polymorphism might play a role in disease susceptibility. Evidence was provided by Kotb and coworkers [90], who found that SPE-A triggered significantly higher proliferative responses when presented by HLA-DQ compared to HLA-DR1, HLA-DR4, or HLA-DR5 alleles. In contrast, SPE-C was preferentially presented by DR4. Moreover, patients with the DRB1*1501/DQB1*0602 haplotype mounted significantly reduced responses to streptococcal SAgs and were less likely to develop severe systemic disease compared to individuals with risk or neutral haplotypes [91]. The dependence of SPE-A on HLA-DQ α-chain polymorphism was demonstrated by Llewelyn et al. [92], who showed that SPE-A binds stronger to HLA-DQA1*01 than to HLA-DQA1*03/05 and that differential HLA-DQ binding results in quantitative and qualitative differences in T cell proliferation, cytokine production, and Vβ-specific changes in the T cell repertoire.

Acute Rheumatic Fever

ARF is a postinfection sequelae and the leading cause of preventable pediatric heart disease. It usually occurs in school age children and young adults after pharyngeal infection with *S. pyogenes.* ARF is a cross-reactive immune response to the host's cardiac tissue, and it has been proposed that the reactive T cells might be driven by SAgs. Recently, several novel streptococcal SAgs have been identified from ARF-associated serotypes. The genes for SPE-K/L were found in high frequencies on serotypes M3 (USA and Japan) [41, 42] and M89 (New Zealand) [43], while SPE-L/M and SPE-M were found in M18

(USA) [44]. It was shown that antibodies against SPE-L/M and SPE-M were more common in convalescent sera from ARF patients compared to patients with pharyngitis [93]. Interestingly, a common target of the SAg SPE-K/L, SPE-L/M, and SPE-M are T cells bearing the TCRs with Vβ1.1.

Kawasaki Disease

Kawasaki disease (KD) is an acute multisystem vasculitis of unknown etiology that affects mostly young children and is now recognized as the leading cause of acquired heart disease in children in the developed world. Although KD has been reported all over the world, it is overexpressed among Asian populations, especially Japanese [94].

KD is associated with marked activation of T cells and monocytes, and there is a remarkable similarity among KD, staphylococcal toxic shock, STSS, and scarlet fever in the clinical symptoms. Intravenous immunoglobulin therapy is highly effective when given early, suggesting that the causative agent is a toxin. Several investigators reported the selective expansion of T cells bearing the Vβ2.1TCR, which points towards an SAg involvement in the disease [95, 96]. Elevated plasma levels of IL-1β, IL-2, IL-6, IL-8, IL-10, IFN-β, and TNF-α were observed in the acute phase of KD and levels of anti-SPE-C antibodies were significantly higher in patients with acute and convalescent KD than in age-matched controls [97].

Why Do Bacteria Produce Superantigens?

After more than a decade of intensive research, it is still largely unknown, why bacteria produce toxins that stimulate the immune system. The evolutionary advantage of SAg production, however, seems eminent. Currently, there are 40 staphylococcal and streptococcal SAgs that, despite their often minor sequence homology, share a common protein fold and target the same host cell receptors, MHC class II and TCR. The wide allelic variation observed for SMEZ, which results in antigenic variation rather than functional differences (all SMEZ variants target the same Vβ8 TCR), confirms that the evolution of SAgs is driven by host immunity [37]. A possible advantage conferred by SAg production might involve the corruption of the host immune system. SAgs interfere with the adaptive immune response driving profound Th1-type responses characterized by high levels of type 1 cytokines, such as IL-2, IFN-γ and TNF-α, and nonspecific T cell proliferation. One might argue that the prime function of SAgs is to suppress a Th2-type response thereby preventing

the production of high-affinity cytotoxic antibodies by promoting a Th1-mediated response.

Another possible mechanism of how SAgs corrupt the immune response might be through their ability to cause T cell anergy. Systemic stimulation of T cells by SAgs results in a nonresponsive (anergic) state, which can be reversed in vitro by application of IL-2. Since anergic T cells also fail to produce IL-2 [98], SAg stimulation might also lead to local IL-2 deficiency, which in turn could limit the expansion of antigen-specific T cells [17].

Acknowledgement

The authors are supported by the Health Research Council New Zealand.

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Marone G (ed): Superantigens and Superallergens.
Chem Immunol Allergy. Basel, Karger, 2007, vol 93, pp 24–41

Diversity in *Staphylococcus aureus* Enterotoxins

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Abstract

The molecular mechanism of *Staphylococcus aureus* phathogenicity is complex and involves several toxins, including the famous staphylococcal enterotoxin (SE) and toxic shock syndrome toxin-1 (TSST-1). Although these toxins were discovered in specific clinical contexts of food poisoning and menstrual toxic shock syndrome, they share common biochemical and biological properties. As superantigens they are able to massively activate mononuclear cells and T cells regardless of the antigenic specificity of the T cells. To date, 19 different enterotoxins and related toxins have been described in *S. aureus* with some differences in structure and biological activity. It has been clearly demonstrated that most human *S. aureus* isolates harbor at least one gene encoding for these toxins. It is suspected that *S. aureus* produces SEs and TSST-1 in humans from colonization to infection, whatever the clinical situation. It is proposed that the production of SEs plays a role not only in classical staphylococcal infections but also in noninfectious diseases. This review will focus on recent findings related to staphylococcal superantigens and their impact on human diseases.

Staphylococcus aureus Enterotoxin Discovery

Although the clinical manifestations of staphylococcal infections, including toxemia, were recognized long ago, they were not associated with cocci until the end of the 19th century by several German scientists. The organism was observed in different samples from patients who died of pyemia. Sir Alexander Ogston and Louis Pasteur were able to quickly reproduce the disease observed in humans when they inoculated animals with pus from patients. Ogston further differentiated the organism from *Streptococcus* and gave it the name *Staphylococcus* [1], and in 1884, Rosenbach [2] described and classified the genus *Staphylococcus*.

The first recorded staphylococcal food poisoning outbreak occurred a few years after 1884. It was attributed to the consumption of Cheddar cheese in Michigan [3]. Macroscopic examination of the cheese revealed the presence of spherically shaped bacteria, and following ingestion of them, Vaughn [3] developed symptoms of the illness. The first well-documented report, which clearly identified a *Staphylococcus aureus* toxin as the cause of food poisoning outbreaks, was done by Dack et al. [4]. They isolated a pigment-forming *Staphylococcus* present in large numbers in a Christmas cake responsible for a food poisoning incident, and sterile filtrate from a broth in which the organism was grown produced illness when ingested by human volunteers. Fortunately, it did not take long for Jordan [5] to discover that oral administration of toxic staphylococcal preparations induced vomiting in monkeys as well. In addition to the kitten test devised by Dolman et al. [6], these were the only systems available by which to study staphylococcal enterotoxins (SEs) until the introduction of more 'modern' biochemical and immunological tools in 1950 by Surgalla et al. [7, 8]. They were able to show that SEs were proteins and purified them. Moreover, using sera obtained from rabbits that were injected with enterotoxins produced by several different enterotoxigenic strains of *S. aureus*, Casman [9] demonstrated that there existed several serological forms of enterotoxin.

Description and Properties of Enterotoxins

Initially, five antigenic variants of *S. aureus* SEs designated SEA through SEE were identified and reported in the literature [9–13]. The existence of a sixth toxin referred to as SEF was alluded to, but the toxin was thought to be nonemetic in monkeys. Subsequently, this toxin became famous in the 1980s because it was shown to be responsible for menstrual toxic shock syndrome (TSS) [14, 15]. Since then, new variants have been identified and designated SEH to SE*l*R, and SE*l*U in the order that they were discovered [16–25]. Interestingly, some of these new toxins were reported to lack emetic properties, raising the question as to whether they should be categorically included with the SEs (table 1). It has been proposed that these toxins and toxins which have not yet been tested for emesis in primates should be designated as staphylococcal enterotoxin-*like* (SE*l*), indicating that their potential role in staphylococcal food poisoning remains unconfirmed [26].

Chemical Properties of Staphylococcal Enterotoxin-Related Toxins

Enterotoxins and related toxins are short, secreted proteins (from 194 to 245 AA) soluble in water and saline solutions. They share common biochemical

Table 1. General properties of SEs and TSST-1

Toxin	Molecular mass, Da	pI	Crystal structure determination	Detection in nasal isolates[1], %	Emetic activity	HLA specificity	Human TCR specificity
TSST-1	22,049	7.2	yes	12	no	HLA-DR	hVβ 2.1
SEA	27,100	6.8–7.3	yes	10	yes	HLA-DR	hVβ 1.1; 5.3; 6.3; 6.4; 6.9; 7.3; 7.4; 9.1; 16; 18; 21.3; 22
SEB	28,336	8.6	yes	18	yes	HLA-DR	hVβ 3.2; 6.4; 12; 13.2; 14; 15; 17; 20; 22
SEC	27,496	7	yes	7	yes	HLA-DQ	hVβ 3; 6.4; 6.9; 12; 15.1; 17; 20
SED	26,900	7.2	yes	9	yes	HLA-DR	hVβ 1.1; 5.3; 6.9; 7.4; 8.1; 12.1
SEE	26,425	8.5	no	0	yes	HLA-DR	hVβ 5.1; 6.3; 6.4; 8.1; 18; 21.3
SEG	27,042	8.4	no	53	yes	ND	hVβ 3; 12; 13.1; 13.2; 13.6; 14; 15
SHE	25,145	5.7	yes	5	yes	HLA-DR	hVα 10
SEI	24,928	8.7	no	49	weak	ND	hVβ 1.1; 5.1; 5.2; 5.3; 6b; 23.1

SEJ	28,500	8.8	no	10	yes	ND	ND
SE/K	26,000	7–7.5	no	9	ND	ND	hVβ 1; 5.1; 5.2; 5.3; 6.7; 21.3; 23
SE/L	26,000	8.2	no	7	no	ND	hVβ 5.1; 5.2; 5.3; 6.7; 7; 9; 16; 22; 23
SE/M	24,800	6	no	44	ND	ND	hVβ 6a; 6b; 7.1; 8; 9; 18; 21.3
SE/N	26,100	6.2	no	52	ND	ND	hVβ 5.1; 5.3; 9; 20
SE/O	26,700	5.8	no	43	ND	ND	hVβ 5.1; 7; 21.3
SE/P	27,000	6.6	no	27	ND	ND	hVβ 5.1; 8; 16; 18; 21.3
SE/Q	25,000	6.7	no	7	ND	ND	hVβ 2.1; 5.1; 6.7; 21.3
SE/R	27,000	8.6	no	9	ND	ND	hVβ 3; 5.1; 8; 11; 12; 13.2; 14
SE/U	27,100	6.2	no	ND	ND	ND	hVβ 12; 13.2; 14

Data compiled from the references and personal data. ND = Not determined or reported.
[1]See Omoe et al. [56].

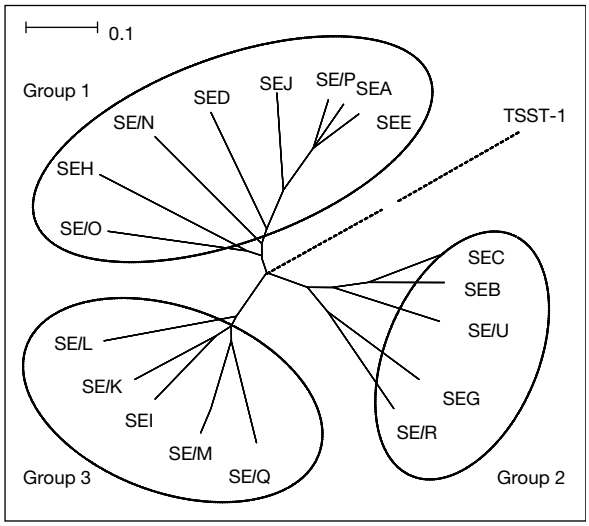

Fig. 1. The SE family tree. This tree has been constructed using Clustal X and SplitsTrees version 4 programs and is based on a multiple amino acid sequence alignment of the mature form of *S. aureus* toxins. The bacterial toxins tree can be divided into four distinct groups, the fourth is only composed of TSST-1.

and structural properties (table 1). Remarkably resistant to heat, the potency of these toxins can only be gradually decreased by prolonged boiling or autoclaving [27]. Excepting toxic shock syndrome toxin-1 (TSST-1), they are highly stable and resistant to most proteolytic enzymes, such as pepsin and trypsin, and thus retain their activity in the digestive tract after ingestion [28]. SEs and TSST-1 display significant homology in their primary amino acid sequences. Based on this sequence comparison, SEs can be divided into four groups as described in figure 1. Crystal structures have been established for SEA, SEB, SEC, SED, SEH and TSST-1 [29–34]. They are all compact ellipsoidal proteins folded into two domains, A and B, which are composed of a mixed α/β structure (fig. 2).

The smaller domain of the SEs and TSST-1, domain B, contains most of the N-terminal half of the protein excluding the N-terminus. Interestingly, domain B resembles members of the oligosaccharide/oligonucleotide binding family. This family also includes the B subunits of the AB(5) heat-labile enterotoxins, cholera toxin, pertussis toxin, and verotoxin [35]. There is no evidence that SEs bind to carbohydrates, but Vojtov et al. [36] have recently shown that TSST-1 and SEB act as negative global regulators of exoprotein gene transcription. However, when they mapped the active domain through the use of truncated toxins, they could not confirm that the transcription-regulator region

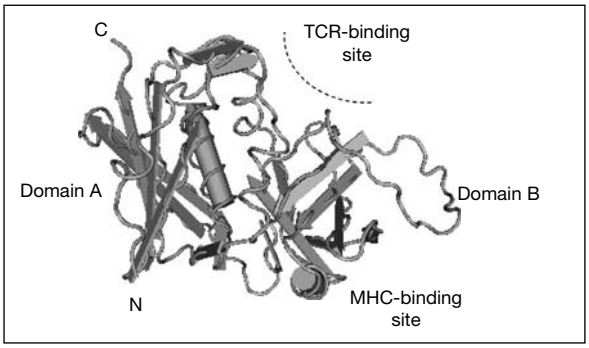

Fig. 2. Ribbon diagram of SEB structure as representative of SEs and related toxins. The diagram has been constructed using Cn3D version 4.1 program. SEs and TSST-1 have a two-domain structure, with a solvent-accessible α-helix in the center. The MHC class II and TCR recognition sites are marked [30].

of these toxins was in the B domain. The top of domain B in SEs also contains a disulfide loop, a conserved feature in all known SEs. The larger domain A has a β-grasp motif and is composed of C-terminal residues as well as the N-terminus, which extends over the top of SEs. This resembles the immunoglobulin-binding motif found in streptococcal proteins, G and L [35]. The interface between the two domains is delineated by a short, shallow cavity at the top of the molecule and by a large groove extending all the way along the back of the molecules. The strong structural homology between the SEs and other bacterial proteins suggests that they evolved through the recombination of two smaller β-strand motifs [35].

Biological Properties of Staphylococcal Enterotoxin-Related Toxins

SEs and TSST-1 share common biological properties. These include induction of high fever similar to bacterial endotoxin induction [37], lethal shock in animals resulting from excessive intravenous doses [38], enhanced host susceptibility to endotoxin lethality [39], cytokine production [40], and polyclonal T-lymphocyte proliferation as seen with superantigens (SAgs) [41].

In contrast with conventional antigens, SEs and TSST-1 interact uniquely with the immune system. As SAgs, they can directly bind, unprocessed, to major histocompatibility complex (MHC) class II molecules on antigen-presenting cells (APCs) (fig. 2). Briefly, the β-barrel of the SE domain A binds to the MHC II at a region distinct from the peptide-binding groove [30, 42, 43]. The toxins are able to bind a wide variety of molecules, although there is some preference depending

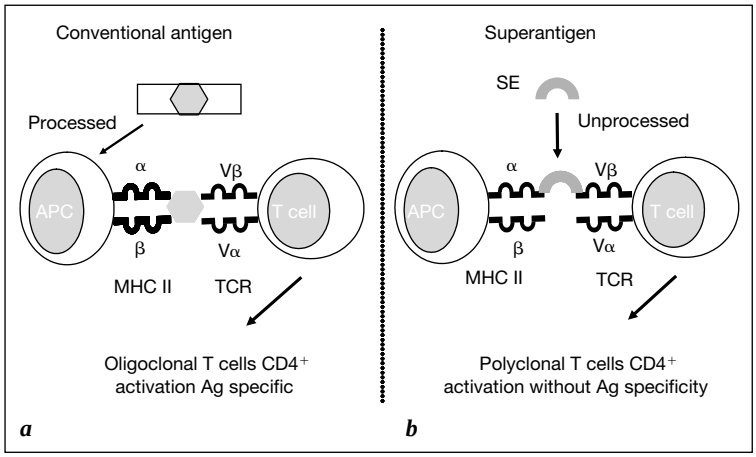

Fig. 3. Schematic representation of T cell stimulation by antigen (***a***) and SAg (***b***).

on the toxin involved (table 1). SEC prefers HLA-DQ and SEA, SEB, SED, SEE, SEH and TSST-1 prefer HLA-DR [44]. Finally, the α-helices of domain A can co-bind and facilitate ligation between MHC II molecules of APCs and certain T cell receptors (TCRs) (fig. 2). This binding is not dependent on T cell antigenic specificity but occurs rather as a function of the variable region of the TCR β- or α-chains (fig. 3) [30, 45]. Each SAg interacts with a defined TCR repertoire determined by the TCR Vβ sequences and the residues found in the top shallow cavity of the toxin [46], except SEH which interacts with a sequence of TCR Vα (table 1) [45]. In contrast to antigen-specific interactions with MHC II and TCR, which stimulate approximately 1 in 10,000 T cells, SAgs such as TSST-1 stimulate T cells bearing Vβ2, potentially activating 10–50% of all T cells (fig. 3) [47]. Facilitation of APC-T cell interaction by antigens and/or SAgs activates the same signal transduction pathway, ultimately leading to T cell division and cytokine production by both cell types. However, the massive cell activation induced by SAgs results in the release of various cytokines at high levels from both macrophages and T cells. These include IL-1 and tumor necrosis factor-α (TNF-α) from macrophages and TNF-β, IL-2 and interferon-γ from T cells [48].

SAgs can also cause T cell and B cell immunosuppression by different mechanisms. Prolonged exposure or subsequent stimulations by SEB induced Fas/Fas ligand-mediated apoptosis in T cells [49] and anergy in which they fail to proliferate or secrete IL-2 [50]. The establishment of peripheral T cell anergy by SEB inhibits the specific antigenic priming of helper T cells in vivo but does not prevent primed T cells from helping B cells to mount an effective antibody response [51]. In contrast, high doses of SAg promote B cell apoptosis and the

downregulation of immunoglobulin-secreting cells [52]. Immunosuppression induced by SAg may be demonstrated in vitro and in vivo in patients. For example, recurring TSS observed in patients has been associated with a failure to generate neutralizing antibodies [53]. Thus, it has been proposed that *S. aureus* benefits from immunodepression induced by SEs and TSST-1.

Distribution of Staphylococcal Enterotoxins in Humans

S. aureus is present in the nasal vestibule in at least 30% of the normal population, and this carriage is a major risk factor for infection [54]. The *S. aureus* infections can be schematically categorized into those associated with clinical signs of infection, such as soft tissue infections, abscesses or sepsis due to the pyrogenic potential of the bacteria, and those implicating particularly toxin-based effects, such as in the case of TSS [55]. For a long time, the production of SAgs by *S. aureus* was considered a rare phenomenon linked to the occurrence of staphylococcal toxemia and therefore restricted to isolates responsible for such syndromes. However, when we examined the presence of SEs and TSST-1 in human *S. aureus* isolates, almost all the strains produced one of the SAgs, whatever the clinical context [21]. For example, Omoe et al. [56] showed that 80% of human nasal isolates harbor at least one SAg gene with a distribution as shown in table 1. In fact, SAg production by *S. aureus* isolates appeared more frequent than initially suspected, and it is more common for *S. aureus* to possess an SAg gene than not. Moreover, most of the strains harbor several SAg genes as illustrated in genome sequences of *S. aureus* strains [57].

One recurrent question concerns whether or not non-*S. aureus* staphylococcal isolates from human flora could also produce SEs. There are several publications arguing that non-*S. aureus* staphylococci produce SEs or related toxins, using immunoenzymatic assay methods for their detection (see review in Lina et al. [58]), but this assertion has only been confirmed for *Staphylococcus intermedius* via a T cell mitogenic assay. The genes encoding the SEs produced by *S. intermedius* have now been identified [59]. However, although *S. intermedius* is found in a wide range of animal species, including dogs, cats and pigeons, it has rarely been found in humans. The impact of SEs produced by non-*S. aureus* should be considered negligible.

Staphylococcal Enterotoxin Production and Regulation

S. aureus has developed overlapping subsets of accessory genes dedicated to regulating the expression of other genes, including SEs and TSST-1. The

most well-known and predominant regulon in *S. aureus* is the *agr* system (for accessory gene regulator). Schematically, it is a quorum-sensing system activated at high cell density. In vitro, upon activation, *agr* downregulates the expression of genes encoding surface proteins and upregulates genes encoding exoproteins, such as SEB, SEC, SED and TSST-1 [60, 61]. However, this is not the global rule for SE expression because SEA is constitutively expressed by *S. aureus* while SEG and SEI are produced only at low bacterial concentrations [17], and nothing is known for other SEs.

When are SEs produced by *S. aureus* in humans? It is obvious that SEs and TSST-1 are produced in vivo during TSS and related syndromes. TSST-1 has been quantified in human serum samples from patients with TSS or TSS-like symptoms. Toxin levels up to 5,000 pg/ml (medium of 440 pg/ml) were measured in serum samples [62]. Moreover, in the acute phase, an expansion of TCR Vβ subtype lymphocytes corresponding to the SEs was observed in these patients, and their TCR Vβ subtype lymphocytes were anergic to restimulation by SEs [63]. It has also been argued that in the absence of staphylococcal toxemia, SEs are still produced, even during *S. aureus* colonization. When you examine the level of antibodies against SEs in humans, it increases after the first 3 months of life [64]. The level of antibodies against SEs depends on SE identity but does not correlate with the frequency of SEs [65]. Less than 10% of normal human serum samples inhibited the T cell stimulation elicited by *egc*-encoded enterotoxins (SEG, SEI, SE*l*M, SE*l*N and SE*l*O), whereas between 32 and 86% neutralized the classical SAgs (SEA to SED). The in vivo production of SAgs in humans in the absence of TSS has recently been confirmed by Azuma et al. [66]. They detected circulating SEA, SEB, SEC and TSST-1 in sera from patients with sepsis, although without septic shock. Upon careful examination of the work done by Miwa et al. [62], one also finds TSST-1 in the serum of one healthy blood donor. Unfortunately, in these two studies we do not know whether the donors with toxins detected in their blood were colonized or infected by *S. aureus* strains.

Clinical Significance of Staphylococcal Enterotoxins

Staphylococcal Food Poisoning

Clinical symptoms of staphylococcal food poisoning resulting from ingestion of food contaminated with SEs include nausea, emesis, abdominal pain or cramping, and diarrhea after a short incubation period (mean of 4.4 h) (see review in Tranter [67]). In contrast to the well-known clinical manifestations, the physiopathology of the symptoms is only partially understood. The emetic action of SEs has been studied rather extensively by Sugiyama and Hayama

[68] in the rhesus monkey by testing its emetic responsiveness following destruction of specific neural structures and/or visceral deafferentation. Animals subjected to vagotomy plus abdominal sympathectomy did not vomit even after being administered lethal doses of SEB. Neither vagotomy nor sympathectomy alone were sufficient to block the vomiting reflex, but vagotomy provided increased tolerance to SE. This result indicated that the site of emetic action was in the abdomen and that the stimulus reached the center of the brain responsible for vomiting via these nerves. Gastroscopic examination of patients with acute SE intoxication showed gastrointestinal damage with patchy mucosal hyperemia, regional edema, muscular irritation, erosion, petechiae and purulent exudates [69]. In these areas, one observes an influx of polymorphonuclear cells into the lamina propria and epithelium. SEs can penetrate the gut lining and gain access to both local and systemic immune tissues [70]. It has been postulated that local immune system activation by SEs could be responsible for the gastrointestinal damage observed as well as mast cell activation. The release of inflammatory mediators (histamine and leukotriene) and neuroenteric peptide substance P upon SE activation could be responsible for the gastrointestinal tract damage [71–73]. Histamine release is also associated with other types of food poisoning [74], and the emetic response can be eliminated by H2-blockers and calcium channel blockers, both of which block histamine release [75]. The question still remains as to whether synthesis of these mediators is induced directly or indirectly by SEs.

When examined, the jejunum of these patients shows signs of crypt extension, disruption or loss of the brush border, as well as an extensive infiltrate of polymorphonuclear cells and macrophages into the lamina propria. Diarrhea in staphylococcal food poisoning may be due to the inhibition of water and electrolyte reabsorption in the small intestine by SEs as observed in vitro [76] and in an animal model [77].

Emetic activities of SEs and related toxins are not equivalent (table 1). Since the initial description of SE antigenic variants in the 1960s, differences have been observed. SEF, also known as TSST-1, is not emetic in the monkey feeding assay, probably due to its sensitivity to digestive tract enzymes. Other SEs, such SEI and SE*l*L, were shown to be either weak or nonemetic [17, 20]. These two toxins lack the disulfide bond characteristically at the top of domain B. The motif in itself does not appear to be an absolute requirement for either activity; however, disulfide linkage may stabilize a crucial conformation within or adjacent to the loop which is important for emesis activity [78]. Studies involving mutations in various SEs that dramatically reduce T cell proliferation with no demonstrable effect on emesis suggests there is little or no link between the two [79, 80]. Studies that dissociate monocyte activation from emesis are still lacking.

Toxic Shock Syndrome

First reported by Tood [81], TSS is a major systemic illness associated with acute intoxication with TSST-1 and SEs. It is characterized by an acute onset of high fever, diffuse erythematous rash, desquamation of the skin 1–2 weeks after onset (if not fatal), hypotension, and the involvement of three or more organ system failures (see review in Todd [81]). During the 1980s, an epidemic of TSS was observed among menstruating women who were using tampons, particularly those of higher absorbency. The illness was associated with the presence of toxigenic *S. aureus* strains producing TSST-1 [14, 15]. Out of the SAgs, TSST-1 is the only toxin responsible for menstrual TSS because it is the only SAg capable of traversing the vaginal mucosa. Since then, tampons made of polyacrylate instead of cotton have been removed from the market. The number of menstrual TSS cases has dramatically decreased and has become lower in frequency than nonmenstrual cases [82]. Additionally, middle forms of TSS have been described [63, 83]. Toxins involved in nonmenstrual TSS include not only TSST-1 but also SEs, especially SEA, SEB and SEC [84].

The TSS pathophysiology model involves a capillary leak syndrome stemming from toxin- and cytokine-mediated endothelium damage. In vitro, SAgs as well as proinflammatory cytokines are lethal for endothelial cells [85, 86]. Differences in the proinflammatory behaviors of SAgs have been suggested in several epidemiological studies. Ferry et al. [87] showed a higher prevalence of *sea* genes for *S. aureus* strains in septic patients experiencing shock than in patients without shock. Similarly, Peacock et al. [88] showed an association between the *sea* or *sej* genes and strains coming from invasive samples. In contrast, *egc* strains that produce SEG, SEI, SE*l*M, SE*l*N and SE*l*O were more pervasive in isolates from suppurative infections than from invasive diseases, suggesting a negative association between the presence of *S. aureus* strains with the *egc* cluster and severity of the patients' conditions [87]. Regarding the respective roles of monocytes and T lymphocytes in the host inflammatory response to SAgs, the literature remains controversial. Dinges et al. [89] showed T-independent mortality induced by TSST-1 in a rabbit model. SEB induced the production of TNF-α by isolated monocytes but not by nonmonocytes or purified T cells, suggesting that the proinflammatory reaction can occur without the presence of T cells [90]. On monocytes, SAgs interact mainly with MHC class II. SEA, SED and SEE possess a cysteine domain involved in high-affinity binding to MHC class II that is absent in SEB, SEC and TSST-1 [32, 91]. Thus, SEA and SEs with a cysteine domain bind two sites on the class II molecule – one high-affinity site and a second low-affinity site. In contrast, non-SEA-related toxins appear to bind only the α-chain of MCH class II with low affinity [30, 92]. It has been hypothesized that differences in SAg inflammatory properties could be a reflection of respective differences in their MHC class II binding

properties. Grossman et al. [93] showed that both the SEA- and the TSST-1-induced monocyte production of TNF-α could be significantly blocked by anti-MHC class II antibodies. The study indicates that TNF-α production by monocytes generally correlates with the binding of the toxin to MHC class II. Interestingly, Wright and Chapes [94] proposed another mechanism of action for SAgs on monocytes. They demonstrated that SEA could also interact with MHC class I on monocytic cells deficient for MHC class II and induce an important cytokine response, most notably the release of TNF-α and IL-6.

On the other hand, several reports proposed a predominant role for T lymphocytes in inducing the inflammatory response. Marrack et al. [95] showed that the pathological effect of SEB may be the consequence of massive T cell stimulation. They demonstrated that T-cell-deficient mice were less susceptible to the toxic effect of SEB than normal mice. Also, Faulkner et al. [96] have recently demonstrated that the α/β TCR was critical for toxic shock lethality using an HLA-DR1 transgenic mouse model.

Lastly, it has been suggested that the synergy between Gram-negative endotoxins and SAg contributes to TSS lethality through TNF. The administration of TSST-1 in rabbits enhances their susceptibility to endotoxins by 50,000-fold [97]. Moreover, they observed that when the rabbit was challenged with TSST-1, endogenous lipopolysaccharide (LPS) levels increased probably due to interference with hepatic clearance [98]. The prediction that SAg is hepatotoxic has been confirmed [99]. The molecular mechanism of endotoxin and SAg synergy is now understood. In vitro, SAg-MHC II interaction and INF-γ secreted by SAg-activated T cells induced an increase in the expression of TLR4, the cell receptor of LPS [100, 101]. In the same way, LPS enhanced MHC II expression on APC [102].

Noninfectious Disease

It has been proposed that SAgs might contribute to the pathogenesis of noninfectious diseases by activating T cells that are specific for self-antigens. Early reports observed Vβ-specific enrichments of T cells in different diseases, such as Kawasaki's disease (KD) and autoimmune disease.

KD is an acute febrile disease in children that resembles TSS [103], although the etiological agent remains unknown. Intravenous immunoglobulin therapy is highly effective when given in early stages of the disease, suggesting the responsible agent is a toxin that can be neutralized by naturally occurring anti-toxin antibodies. Leung et al. [104, 105] found that most of the patients were colonized with TSST-1 and exhibited selective expansion of a Vβ2 subset of T cells. Moreover, some patients increased their levels of IgM antibodies against SAg in the weeks following diagnosis with KD [106]. Further investigations have, however, not been able to substantiate the connection between SAg and KD.

Similarly, early reports found a selective expansion of Vβ subsets of synovial T cells in arthritis [107] and in the pancreas of insulin-dependent diabetes mellitus [108]. In an animal model for multiple sclerosis, it has been shown that SEB has detrimental effects on the disease [109].

Recently, it has also been postulated that SAg could play a role in skin and airway allergies. It has been well investigated in atopic dermatitis (AD). Zollner et al. [110] found that clinical severity corresponded with the colonization of toxin-producing *S. aureus* when they focused on SEA and SEB. TCR Vβ repertoire of the skin-infiltrating T cells in AD has been correlated with the pattern of toxins produced by the individual *S. aureus* strains [111]. At least IgE antibodies directed against SEA and SEB were detectable in AD patients, and their levels were associated with the disease, suggesting that SAg could function as conventional allergens [112]. Again, further investigations were unable to confirm the connection between SAg and AD.

In fact, there is little evidence that clearly and directly links SAg to all these diseases, although SAg can and does play a secondary role in immune activation through the relatively indiscriminate stimulation of autoreactive T cells.

Conclusion

The toxins described in this review constitute the most efficient T cell stimulators known. Because of the ubiquitous production of SEs by *S. aureus*, it has been proposed that these toxins play a role not only in classical staphylococcal toxemia but also in noninfectious diseases. A current working hypothesis is that the human immune system is constantly being challenged by these powerful toxins. Despite the shared characteristic of facilitating TCR and MHC II ligation, SEs and TSST-1 display a remarkable degree of variation in structure and function. The effect of SE variability on the host as well as its advantages for the bacteria remains an open question.

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Marone G (ed): Superantigens and Superallergens.
Chem Immunol Allergy. Basel, Karger, 2007, vol 93, pp 42–57

The SaPIs: Mobile Pathogenicity Islands of *Staphylococcus*

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Abstract

The SaPIs are 15- to 17-kb mobile pathogenicity islands in staphylococci. They usually carry two or more superantigens and are responsible for most superantigen-related human diseases, especially staphylococcal toxic shock syndrome. SaPIs are extremely common in *Staphylococcus aureus*, with all but one of the sequenced genomes containing one or more. The SaPIs have a highly conserved overall genome organization, parallel to that of typical temperate phages. Each occupies a specific chromosomal site from which it is induced to excise and replicate by one or more specific staphylococcal phages. Following replication, the SaPI DNA is efficiently encapsidated into infectious small-headed phage-like particles, resulting in extremely high transfer frequencies.

Pathogenicity islands have recently been recognized as the repository of virulence genes in many organisms [1, 2]. Although genetic analyses indicate that they have been acquired by horizontal transfer, and that their acquisition is clearly responsible for the conversion of weakly pathogenic or nonpathogenic enteric organisms into major pathogens, their mobility is generally impossible to demonstrate. This has suggested that following acquisition, they have become transfer defective owing to deletions, mutations or other genotypic rearrangements.

The situation in staphylococci is somewhat different. The first staphylococcal pathogenicity islands (SaPIs) identified (SaPIs 1, 2, and 3) were discovered because of their carriage of the genes for toxic shock syndrome toxin-1 (TSST-1) and other superantigens. They were soon found to be highly mobile, owing to their relationship with certain phages [3]. The SaPIs are inserted at specific

chromosomal sites (att_S) and each is always in the same orientation. With the exception of SaPIbov2 (27 kb), the functional ones are approximately 14–17 kb; a highly degenerate one, of 3.14 kb, is present in 5 of the sequenced genomes. This chapter summarizes the current understanding of the SaPIs, of which complete sequences are known for 20, and which form a highly coherent family with conserved functional and genetic organization. Several other putative pathogenicity islands have been described on the basis of genome sequencing and other studies; these are 25–30 kb in length, encode sets of enterotoxin-like, protease-like or lipase-like proteins, restriction-modification systems and/or transposon remnants [4]. They are rather widespread among *Staphylococcus aureus* strains, mobility has not been demonstrated, and their designation as horizontally transferred chromosomal islands is inferential. They are not detailed here, since this chapter is concerned exclusively with the SaPIs. While coliphage P4 (see review in Lindqvist et al. [5]) and *Sulfolobus* plasmid pSSVx [6] share several properties with the SaPIs, such elements have yet to be described in other Gram-positive bacteria.

Nomenclature

'SaPI' (staphylococcal pathogenicity island) was initially used to designate chromosomally located mobile elements in staphylococci on the basis of their superantigen content [3]. This designation was reinforced when it became clear that the basic core genome organization of these elements was highly conserved and unique (see below). For this reason, it is proposed to continue the use of 'SaPI' for staphylococcal genomic islands that belong to this clearly defined family, rather than the nomenclature proposed by Baba et al. [7] which lumps all known or putative genomic islands together, ignoring the uniqueness of the SaPIs while emphasizing that of the SCCmecs, which are inexplicably excluded. For the purpose of continuity, the designations by Baba et al. [7] are indicated parenthetically. Since insertion site specificity is arguably the defining feature of mobile elements such as these, it is suggested that the designation 'SaPI' be taken to imply site specificity. Previously described SaPIs, including those identified by Kuroda et al. [4] as well as by ourselves and by others [8], would retain their original designations. We suggest that newer elements belonging to the SaPI category be designated SaPIn, where 'n' refers to the numerical order of discovery, or to a strain number. The generic term for the group 'SaPI' would apply even to elements identified in non-*S.-aureus* staphylococci, and the 'a' would be changed accordingly to indicate species. We therefore propose to redesignate SaGlm (type II νSa3) as SaPIm4, and suggest SaPI122 for the newly described second SaPI in strain RF122 (GenBank NC_007622) and SaPI1028

for the element in NY940 initially described as a prophage [9]. We also suggest SaPImw2 for the SaPI in strain mw2, also listed as 'type II νSa3' [7] and SaPI6Δ for the 3.14-kb SaPI remnant present at the 44' site in 5 of the sequenced genomes, and designated as 'type II νSa4'. Since this remnant is present in 5 of the 9 sequenced genomes, a unique strain-specific designation seemed inappropriate so the next available number, '6', has been used. The recently published sequences of *Staphylococcus haemolyticus* [10] and *Staphylococcus saprophyticus* [11] each contain an SaPI, whereas neither of the two known *Staphylococcus epidermidis* genomes [12, 13] contains one. The element in *S. haemolyticus,* νSh2, would be redesignated ShPI2, and νSs15305 from *S. saprophyticus* strain 15305, SsPI15305.

Superantigen and Other Accessory Genes

Staphylococcal superantigens have been found, thus far, only in association with mobile genetic elements, most frequently SaPIs. The biological basis for this is entirely mysterious, but is consistent with the observation that superantigens in other organisms, including not only group A streptococci, but also mice, are carried by mobile genetic elements including phages and retroviruses such as murine tumor virus [14]. The toxin most frequently encoded by SaPIs is TSST-1, which has been found, thus far, only associated with SaPIs; these are therefore exclusively responsible for menstrual toxic shock syndrome. Superantigen and other virulence genes are located at either end of an SaPI, and some are flanked by nonmatching sequences, suggesting that they have been inserted into preexisting genetic units by nonhomologous recombination events. A particularly striking case is that of *tst* (encoding TSST-1) and *seb* (encoding enterotoxin B) which are inserted in opposite orientations at precisely the same site in SaPI1 and SaPI3, respectively, a site that is also present, but unoccupied, in SaPI5.

Many of the SaPIs encode two or three superantigen toxins. Diverse additional accessory genes (i.e., unrelated to the SaPI life cycle) with possible roles in virulence or antibiotic resistance are present in some. Thus several carry the *ear* gene, encoding a penicillin-binding protein homolog which determines penicillin resistance in *Escherichia coli,* others carry a homolog of the *E. coli* ferrichrome ABC transporter, *fhuD,* one carries an exfoliatin A (*eta*) homolog, one carries *bap,* encoding an approximately 2,000-kDa adhesin, one carries a multidrug export homolog (*mdr*), and finally, one carries homologs of aminoglycoside adenyl transferase (*aad*) (streptomycin resistance) and of a metallothiol transferase (*fosB*) (fosfomycin resistance). In most cases, however, the functionality of these genes has not been established.

Staphylococcal Pathogenicity Islands and Their Genomes

The SaPIs are widespread among staphylococci and all of the sequenced strains, with the exception of MSSA476, carry one or more, including several that do not carry any superantigen or other discernable accessory gene. At the time of this writing, 20 have been sequenced, and details of sizes and attachment site sequences are listed in table 1, with designations as noted above (SaPIm1 and SaPIn1 are essentially identical, as are the 5 SaPI6Δs and are not listed separately).

In general, the SaPIs form a coherent group with a highly conserved set of core genes; among these, the integrase, the Rep protein (usually annotated as helicase-like or primase-like), and the terminase small subunit homolog are always present and there is always a characteristic point of transcriptional divergence, near the integrase gene, flanked by two open reading frames (ORFs) encoding transcriptional regulatory proteins. This conserved core genome, transcriptional organization, and mobility mechanism (described in more detail below) serve to separate the SaPIs from all other mobile or potentially mobile elements among the staphylococci. Additionally, most of the other SaPI-specific ORFs do not have close orthologs anywhere in the staphylococcal genomes or elsewhere in the database.

A diagrammatic comparison of the sequenced SaPIs is presented in figure 1, showing the conservation of overall organization and homologies based on similarities to SaPI1. Genes with over 50% sequence similarity to the corresponding SaPI gene at the amino acid level are shown in different shadings. See fig. 1 legend for details. This organization is characterized by two major groups of genes that are divergently transcribed, with the ORFs flanking the divergence shown in dark gray. These core SaPI genes have been partially characterized in SaPI and SaPIbov1 [Barry et al., unpubl. data; Ubeda et al., unpubl. data]. Within the rightward group is a set of highly conserved genes including *ter*, a close homolog of the terminase small subunit of several phages from Gram-positive bacteria, that has predictably been found to be required for encapsidation [Ubeda et al., in preparation]. Five of these genes, ORFs 6–10 in SaPIbov1, are controlled by LexA [Ubeda et al., in preparation] and probably constitute an operon that is provisionally referred to as the encapsidation module. To the left of this module are two or more conserved ORFs of unknown function followed by the replication origin and a gene, *rep*, whose product is similar to helicases and primases. The Rep protein is required for SaPI replication [Barry et al., unpubl. data]; as it is SaPI specific [Ubeda et al., in preparation], it corresponds to the replicon-specific initiator proteins of other types of replicons. This set of genes is provisionally referred to as the replication module. Further to the left and flanking the point of divergence are two small ORFs with similarity to a

Table 1. The SaPI family

Element (strain)	Size, kb(comment)	Inducing phages	*att* site core (location)	Virulence genes	Orientation	References
SaPI4 (MRSA252)	15.1	endogenous prophage	AAAGAAGA ACAATAATAT (~8′)	none	+	20; Subedi and Novick, unpubl. data
SaPI1028 (NY940)	15.6	endogenous prophage	AAAGAAGAAC AATAATAT (~8′)	none	+	9; Subedi and Novick, unpubl. data
SaPIbov1 (RF122 νSa2)	15.8 (excised spontaneously)	ϕ11, ϕ1477, 80α	TAATTATTCCC ACTCAAT (~9′)	*tst, sel, sek*	+	16, 17
SaPIbov2 (V329)	27 (excised spontaneously)	80α	TAATTATTCCC ACTCGAT (~9′)	*bap*		17
SaPIm4 (mu50: SaGIm νSa3 type I)	14.4	endogenous prophage	TCCCGCCGT CTCCAT (~18′)	*fhuD*	+	7; Subedi and Novick, unpubl. data
SaPImw2 (mw2: νSa3 type II)	14.4	endogenous prophage	TCCCGCCG TCTCCAT (~18′)	*ear, se2l, sec4*	+	7; Subedi and Novick, unpubl. data
ShPI2 (*S. haemolyticus,* νSh2)	16.6	ND	TCCCGCCGTC TCCAT (48′)[1]	none	−	10
SaPI1 (RN4282 νSa1)	15.2	80α, Φ13	TTATTTAGCA GGAATAA (~19′)	*ear, tst, sek, seq*	+	3
SaPI3 (COLνSa1)	15.6	29 (?)	TTATTTAGCAG GAATAA (~19′)	*ear, seb, sel, sek*	+	21
SaPI5 (USA300)	14.0	ND	TTATTTAGCAG GAATAA (~19′)	*ear, sek, seq*	+	8

SaPIn1 (n315) SaPIm1 (mu50) (νSa4 type I)	(identical) 15	ND	GTTTTACCATCAT TCCCGGCAT (~44′)	*tst, sel, sec3*	−	4
SaPI2 (RN3984)	14.7	80	ATTTTACATCATT CCTGGCAT (~44′)	*tst, eta*	−	15; Subedi and Novick, unpubl. data
SaPI122 (RF122)	17.9	endogenous prophage	GTTTTACATCAT TCCTGGCAT (~44′)	*mdr*	−	GenBank NC_007622
SaPI6Δ (8325, COL, USA300, MSSA476, mw2: νSa4 type II)	(identical) 3.14 (excised spontaneously)	ND	GTTTTACCATC ATTCCCGGCAT, GTT TTACATCATTCCTG GCAT (~44′)	none	−	7; Novick, unpubl. data
SsPI15305 (*S. saprophyticus* 15305, νSs15305)	17.0	ND	CGAGGGGACTA ATAAGT, CGAGGG GATTAATAAGT (47′)	*aad, fosB*	−	11

ND = No data.

[1] ShPI2 is located 180° away from the other SaPIs having the same *att* core sequence, owing to the major chromosomal inversion that has been documented in the *S. haemolyticus* genome [10].

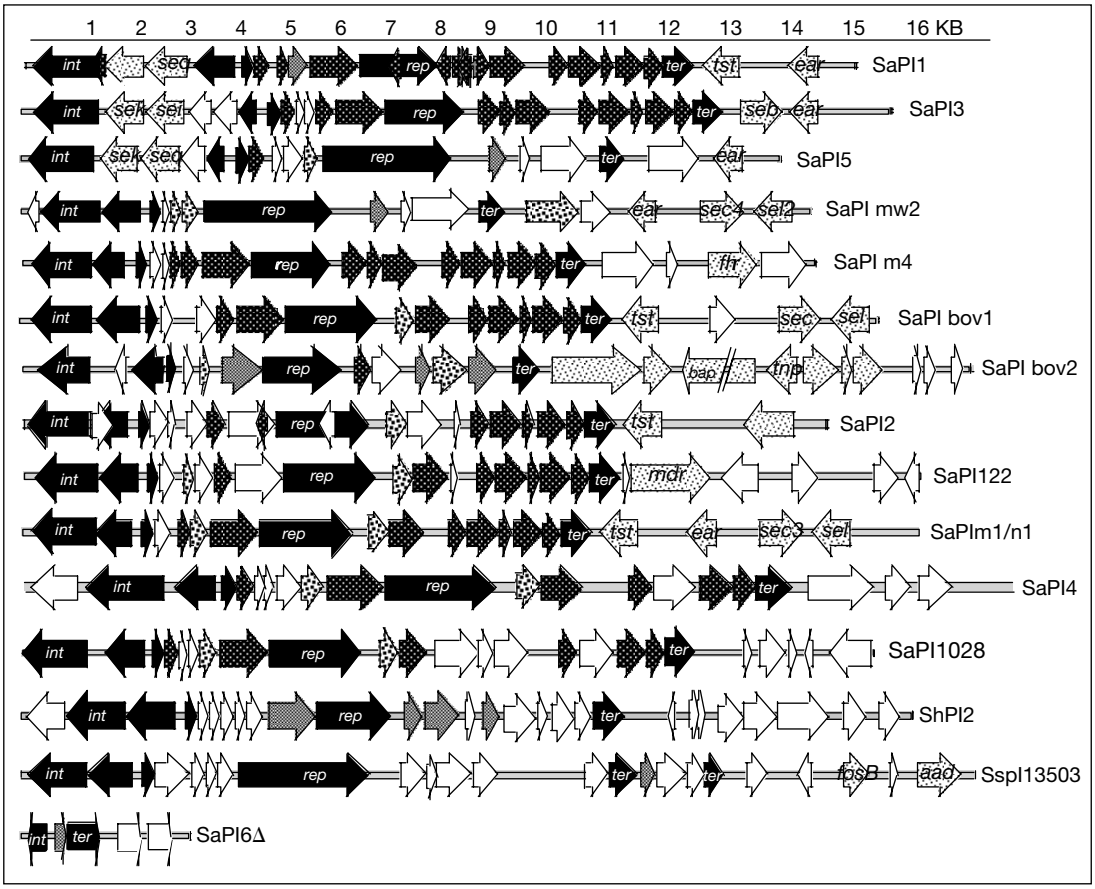

Fig. 1. Comparison of SaPI genomes. Genomes are aligned according to the prophage convention with the integrase gene at the left end. Patterns of ORFs are as follows: genes that are functionally conserved in all SaPIs are shown in black (sequence similarity varies widely); genes that mark the transcriptional divergence are shown in dark gray; most, or all of these, encode transcriptional regulators; genes encoding toxins and other potential virulence or resistance factors are shown as ; genes of unknown function involved in the SaPI life cycle are shown in various shadings, depending on their similarity to the corresponding genes in SaPI1: = >90%; = 50–90%; = 20–50%; = <20%. For SaPI1028, the sequence illustrated here is a permutation of the published sequence. *ter* = Terminase small subunit; *rep* = initiator protein (annotated as 'helicase' or 'primase'); *int* = integrase'; *tnp* = transposase; *sec, sek, sel*, *seq* = enterotoxins C, K, L, Q, respectively; *eta* = exfoliatin A; *ear* = *E. coli* ampicillin resistance; *mdr* = multidrug export protein; *bap* = bovine adhesion protein; *fhuD* = ferrichrome transport protein; *aad* = aminoglycoside transacetylase; *fosB* = fosfomycin resistance.

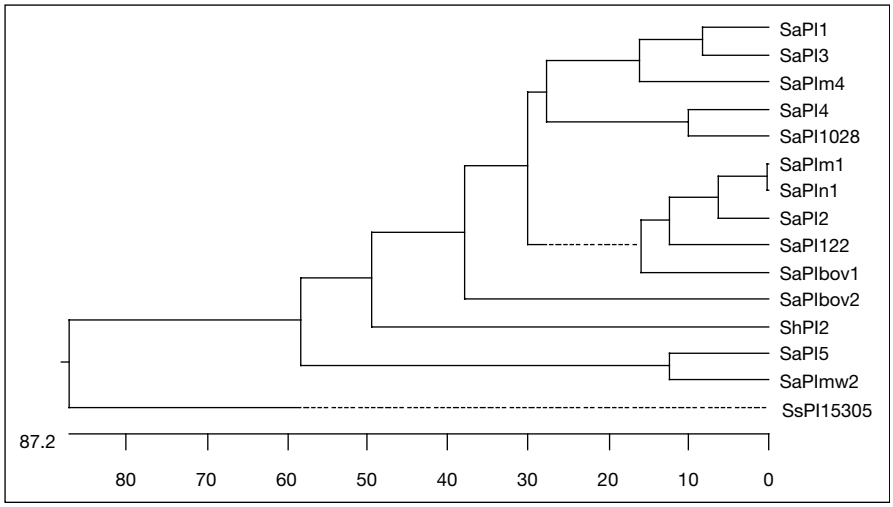

Fig. 2. Nearest-neighbor tree for SaPI core genomes. Lineups were determined and trees calculated by CLUSTAL analysis using VectorNti or MegAlign. Accessory genes were removed from the sequences used for this alignment so as to obtain an accurate view of the relationships among the basic SaPI genomes.

variety of regulatory proteins possessing the helix-turn-helix motif, and presumably representing a regulatory module [Barry and Novick, unpubl. data].

Still further to the left, in several SaPIs, are 2 superantigen genes followed by the integrase gene, which is nearly always adjacent to the *att* site. In several of the SaPIs, the superantigen and other accessory genes are at the other end, and some have superantigen genes at both ends. Although the overall organization of the SaPI genomes is well conserved, two of them, SaPIm4 and SaPImw2, have diverged widely from the rest as exemplified by the nearest-neighbor tree shown in figure 2. Although ShPI2 from *S. haemolyticus* fits nicely into the SaPI family, SsPI15305 from *S. saprophyticus* is a distant outlier, though it contains all of the SaPI-specific organizational features. SaPI6Δ is at the same site as SaPIn1, SaPIm1, SaPI122 and SaPI2, has a *ter* gene that is >94% identical with the other SaPI *ter* genes, has a degraded *int* gene *ψint* consisting of a 96-amino-acid region that contains a frame shift and is 96% identical to the integrases encoded by the other SaPIs at this site, and has a third ORF (D) that is 74% identical with SaPI1 ORF5, a 70-codon ORF that is widely conserved among the SaPIs. This element, presumably derived from SaPIs located at this site, is clearly an SaPI remnant, hence the 'Δ'. Nevertheless, it has two

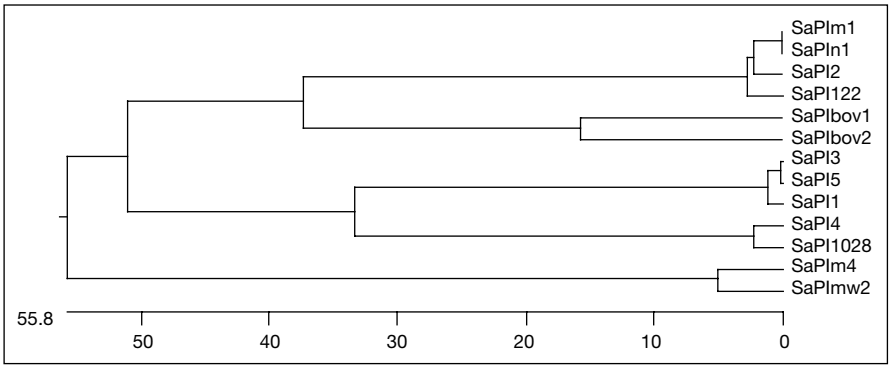

Fig. 3. Nearest-neighbor tree for the SaPI integrases.

other ORFs, A and B, that are not present in any other SaPI or elsewhere in the *S. aureus* genome.

Integration and Excision

Because the complex of integrase and insertion site is highly evolved and not readily amenable to evolutionary modification, whereas specific genetic segments can readily be acquired, exchanged, rearranged or lost, chromosomally located mobile genetic elements such as the SaPIs, SCCmecs, and prophages, that encode and utilize site-specific integrases, are always located at an integrase-determined site and are flanked by directly repeated sequences that represent the core integrase recognition element. As one would expect, any SaPIs occupying the same site have highly similar or identical integrases, though the rest of their genes may vary widely. An unrooted tree for 15 SaPI integrases is presented in figure 3, from which it can be seen that those catalyzing insertion into the same site are always very closely related whereas those acting at different *att* sites are more or less divergent. The SaPI1 integrase is necessary and sufficient for SaPI1 integration [15] but is almost certainly not sufficient for excision since neither SaPI1 nor a plasmid containing only the *att-int* region, integrated at the SaPI1 *att* site, excises spontaneously at a detectable frequency [15]. Spontaneous excision has, however, been reported for several of the SaPIs, including SaPIbov1, SaPIbov2 and SaPI6Δ (table 1). This is based on detection by PCR of the joined SaPI ends; however, the possibility of homologous recombination between the flanking repeats has not always been ruled out, nor has the

possibility of a contribution from an endogenous prophage. The flanking repeats, of 15–20 nt, are probably sufficient to support a low frequency of homologous recombination. Although integrase-catalyzed excision has been demonstrated for an SaPIbov1 clone in *E. coli*, this may not correspond to the situation in *S. aureus*, since it bypasses the question of *int* regulation. In at least one case, that of SaPI1, *int* is not expressed and spontaneous excision is not detectable in the absence of an inducing prophage, which, incidentally, also provides the required *xis* function. In the case of SaPI6Δ, it is difficult to imagine that the highly degraded *int* encodes a functional integrase. Additionally, whether Kuroda et al. [4] observed spontaneous excision for SaPIn1 and m1, which are integrated at the same site and have intact *int* genes, is not clear from their report.

Evolution: Staphylococcal Pathogenicity Island Insertion Sites and Integrases

The known SaPIs occupy 6 different sites in the staphylococcal genome. As shown in figure 4, two pairs of these sites are very close together: those at 8′ and 9′ are within 22 kb, those at 18′ and 19′ are within about 40. Given the diversity of the sequenced strains, it is likely these represent all or most of the SaPI sites in *S. aureus.* Those in the right replichore are all oriented in the clockwise direction (+), those in the left replichore are all counterclockwise (−), as is the case with prophages. Note that ShPI2 is located 180° away from SaPImw2 and SaPIm4, which have the same *att* site core sequence. This is because a very large segment of the *S. haemolyticus* chromosome is inverted with respect to the replication origin [10]. We note that *S. saprophyticus* contains the *att* site found at 44′ in all of the sequenced *S. aureus* genomes, but this site, at the 3′ end of *groEL*, is located at 21′ in *S. saprophyticus*, suggesting that there is a major chromosomal rearrangement in this species as well as in *S. haemolyticus*, as compared to *S. aureus.*

The chromosomal SaPI *att* sites are referred to as att_C, and the matching SaPI site, att_S. As with classical temperate phages, the *att* sites contain a conserved core, of 15–22 nt, plus essential flanking sequences that differ between SaPI and chromosome. The 72-nt direct repeat flanking SaPIbov1 [16] is not conserved; the 56-nt subsequence, AAAAAAGGCTGGAAACCGCGTAATTACGGTAACTCCAGCCTATCATTTGCTATATA, is included, but is not essential for integration [17]. However, an 18-nt subsequence, TAATTATTCCCACTCAAT, which is conserved and at the same site throughout the sequenced strains represents the att_C core for SaPIbov2, and is used for SaPIbov1 integration in other strains, suggesting that SaPIbov1 and 2 insert at the same site and

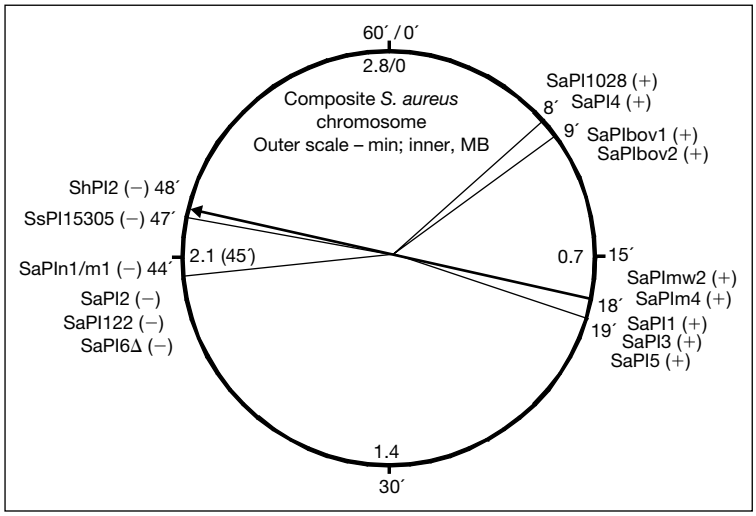

Fig. 4. Locations of SaPIs. The circle shows a composite of the sequenced staphylococcal chromosomes, which all approximate a 2.8-Mbase length. The locations of the SaPI *att* sites were determined by BLAST analysis, using the sequences shown in table 1. Each of the sites is localized to within 100 kb over the set of sequenced chromosomes, and with the exception of *S. haemolyticus*, in which there has been a major chromosomal inversion, the sites are always in the same order. Figures outside the circle are in minutes using average locations and assigning a value of 60′ to the 2.8-Mbase chromosome. Figures inside the circle are in Mbases. Plus or minus after the SaPI designation indicates clockwise or counterclockwise orientation with respect to the chromosome as shown.

that this 18-nt segment is sufficient to serve as the att_C core for both [17]. No SaPI *att* site, however, has yet been fully mapped, except that the SaPI1 sites, att_C and att_S, are each less than 400 nt in length [unpubl. data]. Examination of the published genome sequences has revealed that each of the att_C cores is present in all sequenced *S. aureus* strains, in either 1 or 2 copies and always at corresponding sites. In all cases where there are 2 copies, an SaPI is present between them. Presumably, any of these strains is capable of accepting each of the SaPIs. Note that SaPIm4 occupies a site in Mu50 at which there is no insertion in the closely related strain N315.

Recombination?

A striking example of evolution by probable recombination is SaPI5 [8], located at the SaPI1 site. Its ends are identical to those of SaPI1 and its center to

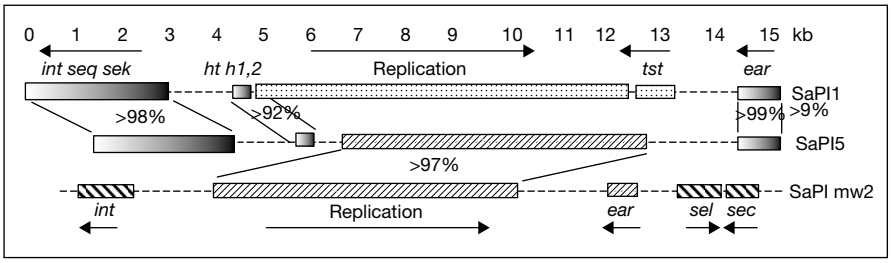

Fig. 5. SaPI recombinants. Diagrammatic representation of SaPIs 1, 5, and mw2, showing nearly identical ends for SaPIs 1 and 5 but highly divergent replication regions, contrasted with highly similar replication regions but unrelated ends for SaPIs 5 and mw2, suggesting that SaPI5 is the outcome of a primary recombination event between SaPIs 1 and mw2 (or similar elements). Percentage figures represent overall sequence similarities at the nucleotide level.

that of SaPImw2 (fig. 5); therefore, its origin must certainly have involved at least one major recombinational exchange. Four of the more recently described SaPIs, SaPI1028, SaPI4, SaPI122 and ShPI2, do not encode any of the known virulence factors or other accessory genes. Very possibly, these represent precursors of the superantigen-encoding mobile elements, as SaPI122 plus the identical elements, SaPIn1 and SaPIm1, are all at the same site (table 1). This site is also occupied in 5 of the 6 sequenced strains by SaPI6Δs. Interestingly, there is a single-base mismatch between the flanking *att* sites of all 5 of the SaPI6Δs, with one copy matching that of SaPIn1/m1, the other that of SaPI122 (fig. 6), suggesting that their evolutionary history probably includes a recombination between these.

Although all of the SaPIs are clearly coancestral, a fascinating question is that of how a highly conserved genome organization might have become associated with different insertion site specificities. The simplest idea is that the genome organization and insertion site specificities evolved independently and were later combined by recombination (fig. 5).

Staphylococcal Pathogenicity Island Life Cycle

The basic life cycle of a prototypical SaPI element such as SaPI1 (see review in Novick [18]) is initiated by phage-induced excision. This requires the SaPI-coded integrase and a phage-determined *xis* function; excision is presumed by the Campbell mechanism since the predicted circular monomer can be detected by PCR [Mathews and Novick, unpubl. data]. However, it cannot

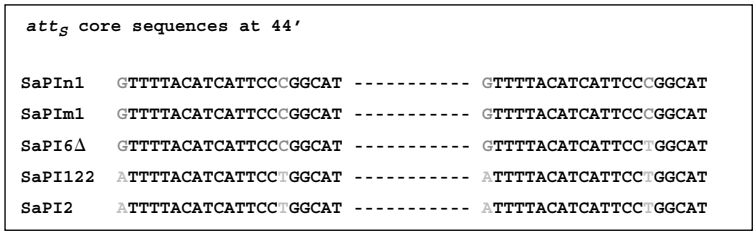

Fig. 6. *att* core sequences for the SaPIs located at 44′.

be detected by gel electrophoresis, including Southern blot hybridization, suggesting that it may be very short-lived. It is suggested that the SaPI replication cycle, like that of satellite phage P4, which mimics that of its helper phage P2, also mimics that of its helper phage. The known helper phages, with one exception, are generalized transducing phages and therefore use the *pac*-dependent headful packaging mechanism. Whether they circularize following injection is not known; however, as linear SaPI1 genomes injected by phage-like particles do not detectably circularize, it is very likely that replication of both SOS-induced and incoming SaPI1 genomes is initiated on linear DNA. Replication is initiated at a unique origin, characterized by a series of hexa- to octanucleotide repeats, by the SaPI-coded helicase-primase-like protein [Barry et al., unpubl. data], possibly analogous to the α-protein of satellite phage P4, and is continued by an unidentified replicase, presumably the host replicase, forming a multimeric complex of unknown structure, presumably similar to that of the replicating phage DNA. Several hundred copies of SaPI DNA are produced, along with a similar or possibly smaller number of phage DNA replicas. During this process, the replicating SaPI DNA comigrates with the chromosomal and replicating phage DNAs. An SaPI-specific band soon appears in the gel electrophoretic pattern, indistinguishable from that seen immediately following DNA injection by infecting SaPI1 particles and therefore representing monomeric linear SaPI DNA that has probably been released from phage heads. Toward the end of the lytic cycle, SaPI DNA can proceed along either of two pathways. It can be circularized, followed by integration, or it can be packaged into small-headed phage-like particles, whose head size is about 1/3 that of the plaque-forming particles, commensurate with the relative sizes of the two genomes, which approach and sometimes surpass the numbers of plaque-forming particles [15]. Packaging requires the conserved SaPI-coded terminase small subunit which presumably acts at an unidentified *pac* site, cleaving the multimeric DNA and conducting it to the phage-coded large

terminase subunit, which would act in conjunction with the portal protein to thread the DNA into capsids. It is likely that the SaPI capsids are formed from the same capsomeres as the plaque-forming particles and that SaPI gene(s) determine(s) the size of these. Filling of the heads would be followed by *ter*-induced cleavage, generating terminally redundant monomeric SaPI DNA, analogous to that of a typical *pac* phage. Tails are then attached that are indistinguishable from the normal phage tails. The resulting SaPI particles are infectious, giving rise to extremely high transfer frequencies. Following entry, SaPI DNA is site-specifically inserted into its chromosomal *att* site by the Campbell mechanism, utilizing the SaPI integrase. Consequently, integrase mutants must be complemented by a functional integrase in both donor and recipient. The integration efficiency probably approaches 100%, though precise data are not available. None of the SaPIs identified to date is capable of autonomous replication, with the possible exception of SaPImw2, for which Baba et al. [7] have reported the presence of multiple autonomous copies on the basis of quantitative PCR in strain mw2. However, we have demonstrated SOS induction of SaPImw2 as well as of SaPI1028 and SaPIm4 [Subedi and Novick, unpubl. data; Ubeda and Penades, unpubl. data] and the same has been demonstrated for SaPI122 by Ubeda et al. [17] almost certainly representing induction by endogenous prophages. We have previously observed autonomous circular SaPI1 DNA in an 80α lysogen [3] but not in a non-lysogen. SOS induction of prophages by antibiotics such as fluoroquinolones and β-lactams occurs with SaPI-inducing prophages as well as with other prophages, resulting in antibiotic-induced high-frequency SaPI transfer [19]. Whether superantigen expression is also specifically SOS induced remains to be determined. It is interesting that phage ϕ13, a *cos* phage, can induce excision, circularization, and replication of SaPI1, though it cannot produce infective SaPI1 particles [15; Mathews and Novick, unpubl. data], consistent with the headful packaging mechanism used by SaPI-transducing phages.

If the SaPI1 att_S site is occupied by a resident copy, the incoming SaPI inserts, at a low frequency, into either of the hybrid *att* sites, creating a tandem double, which is unstable and breaks down to yield single insertions of either type, with the excised element being unable to replicate and therefore being lost [Subedi and Novick, unpubl. data]. Interestingly, some SaPI integrases can apparently catalyze spontaneous SaPI excision in the absence of vegetative phage [17], others cannot [3]. These excised circles cannot replicate, however, and are lost if not quickly reinserted. Thus, SaPIbov1 and other SaPIs that show spontaneous excision are somewhat unstable, whereas those that do not, such as SaPI1, are not spontaneously lost at detectable frequency [15]. The respective integrases of these two types of SaPI element are highly divergent.

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Marone G (ed): Superantigens and Superallergens.
Chem Immunol Allergy. Basel, Karger, 2007, vol 93, pp 58–76

Protein Fv: An Endogenous Immunoglobulin Superantigen and Superallergen

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Abstract

Protein Fv was isolated and purified by Jean Pierre Bouvet and collaborators in 1990 from stool extracts of patients affected by viral hepatitis B or C. This protein was also identified and purified in biological samples in experimental viral hepatitis. Under normal conditions, protein Fv is synthesized in low concentrations by the human liver, and is increased in biological fluids of patients with viral hepatitis. This protein binds with high affinity to the variable domain of the heavy chains of all human immunoglobulins. Thus, it is an endogenous immunoglobulin superantigen because it binds to human immunoglobulin of the V_H3 family. This protein also acts as a superallergen because it activates human basophils and mast cells through the high-affinity interaction with IgE V_H3+. The mechanism of activation of immune cells by protein Fv represents a new pathogenetic cascade consisting of viral infection, endogenous immunoglobulin superantigen/superallergen production and tissue injury.

Protein Fv was isolated and purified by Jean-Pierre Bouvet et al. [1] in 1990 from stool extracts obtained from 43% of patients affected by viral hepatitis C (HCV) and from 35% of patients with hepatitis B (HBV). This factor (originally called 'protein F') was found in only 6.7% of healthy subjects. The original name soon became 'protein Fv' (for Fv fragment) because Bouvet and Pillot [2] provided evidence that this factor binds to the variable domain of the heavy chain of immunoglobulins. Molecular sieving by high performance liquid chromatography of protein Fv indicated an apparent molecular

mass of 175 kDa, whereas in preparative SDS-PAGE the molecular mass was 85 kDa, which suggested that it was a dimer disrupted under dissociating conditions.

Bouvet et al. [1, 3] characterized the immunoglobulin-binding activity of protein Fv, and demonstrated that it binds to immunoglobulins of various isotypes irrespective of light chains (κ and λ). They also found protein Fv in liver extracts from 2 patients affected by HCV infection and from a transplant donor thereby identifying the cellular source of this protein. The acidic isoelectric charge of protein Fv suggested that this molecule is a sialoprotein. This was confirmed by its sensitivity to subtilisin, pepsin and neuraminidase [1]. These results ruled out the hypothesis of synthesis in the digestive lumen by bacteria because sialoglycoproteins cannot be synthesized by bacteria.

The possibility was also considered that protein Fv is a component of HCV. However, protein Fv was detected in biological fluids of patients suffering from viral hepatitis A, B, C, and E [1, 4]. It is highly unlikely that these unrelated pathogens all synthesize protein Fv. This protein was also identified in experimental viral hepatitis. In fact, it was demonstrated that inoculation of rhesus monkeys (*Macaca mulatta*) with stool extracts from a patient suffering from HCV infection induced liver damage [5]. Interestingly, the experimental infection of HCV in monkeys was associated with an increase in the release of protein Fv. It was concluded that the presence of protein Fv in the liver indicated that it was a product of this organ. Its detection in the stools of viral hepatitis patients suggested an increased release in the digestive lumen during these diseases, whereas under normal conditions this molecule is hidden by its binding to secretory IgA (sIgA) [6].

The specificity of human immunoglobulin-protein Fv interaction was investigated with fragments of chains of human monoclonal immunoglobulins [7]. It was demonstrated that protein Fv binds to the V_H region of all human immunoglobulins, whereas no significant binding occurred with the κ or λ light chains.

These observations led Bouvet et al. [7] to investigate the phylogenetic evolution of the immunoglobulin-binding site of protein Fv, which might involve an important region of the V_H framework. They found that binding of protein Fv to immunoglobulins was conserved in all taxonomic classes: mammalian, avian, reptilian, amphibian, and fish. In contrast, no significant binding was found in mammalian ungulates such as horse, cow, sheep, and goat. Such a conserved reactivity during evolution suggested that protein Fv recognized a framework structure in the variable domain of the heavy chain of immunoglobulins.

In conclusion, protein Fv is released in biological fluids of patients with viral hepatitis [1] and can occur as a free molecule in the gut lumen of patients

with hepatitis A, B, C, and E [6]. It is also detected in biological fluids of monkeys experimentally infected with HCV and viral hepatitis E [5, 6]. Protein Fv binds to the variable domain of the heavy chains of immunoglobulin, irrespective of immunoglobulin class, subclass, and light chain type [7]. A single protein Fv molecule can bind six $F(ab')_2$ fragments of human IgM, IgG, and IgE [1, 3, 7]. Binding of protein Fv to the V_H region of immunoglobulins occurs in a domain external to the conventional antigen-binding pocket [1, 3, 7].

Possible Physiological Role of Protein Fv

sIgA is the first immune barrier against pathogens [8]. In humans, the gut-associated lymphoid tissue consists of a major immune organ containing 70–80% of all immunoglobulin-producing cells, with a daily secretion of approximately 40 mg/kg of body weight of IgA, compared with 30 mg/kg of IgG of the total immune system [9].

Dimeric sIgA are actively transported from the lamina propria to the lumen by transcytosis across epithelial cells after binding to the immunoglobulin receptors. The extracellular moiety of this receptor (called 'SC') remains covalently bound to the IgA in the lumen, rendering sIgA resistant to various proteases. sIgA acts by several mechanisms, namely immune exclusion, clearance of immune complexes, and virus neutralization, followed by conveyance of the antigen-antibody complexes in the mucus stream. These functions of sIgA depend on the polymeric status of the molecule, and production of IgA proteases by pathogenetic bacteria is usually considered a virulent factor [10]. Protein Fv binds to sIgA [1] and does not interfere with the specific immune binding of antibodies to their antigen [3].

Bouvet et al. [6] demonstrated that protein Fv, normally released in stools, is involved in macromolecular complexes with sIgA. They demonstrated that the high-molecular-weight complexes contain a sole protein Fv dimer and six molecules of $F(ab')_2$ fragments. This suggested that protein Fv possesses six binding sites for human immunoglobulins (fig. 1). These authors also demonstrated that in vitro addition of protein Fv to anti-virus or to anti-*Salmonella typhi* antibodies augments agglutination of the corresponding pathogens. Identification of these immunologic properties of protein Fv led to the conclusion that protein Fv could play a physiological role in immune defense in the gut [6].

Activation of the complement cascade is one of the major effector functions of antibodies because it leads to cellular lysis of pathogens and to clearance of

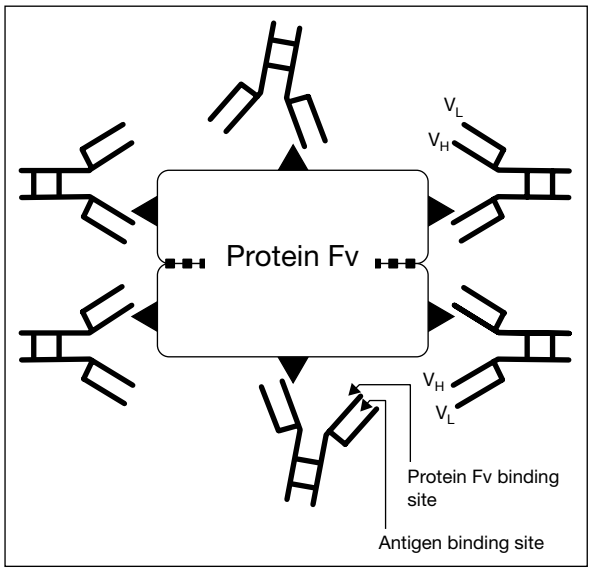

Fig. 1. Schematic representation of protein Fv. This human liver protein has an apparent molecular mass of 175 kDa, whereas in preparative SDS-PAGE the molecular mass was 85 kDa, suggesting that it was a dimer disrupted under dissociating conditions. Protein Fv possesses at least six high-affinity binding sites for human immunoglobulins of all isotypes. Binding of protein Fv to the V_H region of immunoglobulins occurs in a domain external to the conventional antigen-binding pocket. Silverman et al. [22, 23] demonstrated that protein Fv binds with affinity comparable to that of a conventional antigen to immunoglobulin of the V_H3 family.

immune complexes [11]. This multistep complex phenomenon can be triggered by two mechanisms. The classical pathway activates the third component (C3) via activation of the C1, C4, and C2 component, whereas the alternative pathway activates C3 via factor B, factor D and C3 convertase. Triggering of the classical pathway occurs after antigen binding by specific antibodies. Complement activation can be protective against pathogens, but it is also involved in tissue injury due to various mechanisms.

Ruffet et al. [12] demonstrated that preincubation of protein Fv with human monoclonal IgG1 and IgM activates the complement cascade by forming nonimmune complexes. Activation of the classical pathway is due to the binding of immunoglobulins with protein Fv. These results confirm that protein Fv binding mimics antigen-antibody reaction and suggest its involvement in some inflammatory gut diseases [13].

Superantigenic Properties of Protein Fv

Unlike a conventional antigen, which can stimulate less than 0.001% of the naive lymphocyte pool, a superantigen can stimulate greater than 5% of the naive lymphocytes [14]. This immunologic property derives from the unique ability of superantigens to interact specifically with most lymphocytes that express antigen receptors from a particular variable-region gene family. Binding of a superantigen involves antigen receptor sites in the variable regions that are common to members of the variable gene family, but which are spatially distinct from the complementary-determining region sites implicated in antigen binding [15]. Classical superantigens are T cell superantigens (staphylococcal enterotoxins and toxic shock syndrome toxin-1) [15, 16].

Some naturally occurring proteins have the properties of superantigens for B lymphocytes. B cell superantigens are proteins that are endowed with unconventional immunoglobulin-binding capacities that parallel the properties of known T cell superantigens to activate lymphocytes [17]. The best characterized of these immunoglobulin superantigens is *Staphylococcus aureus* protein A which, by virtue of its Fab-binding activity, is considered the B cell superantigen prototype [18, 19]. Other B cell superantigens are the gp120 envelope glycoprotein of HIV-1 [20] and protein L from *Peptostreptococcus magnus* [21].

Silverman et al. [22] investigated the molecular basis for antibody binding of protein Fv. By evaluating a panel of 52 monoclonal IgM, they found that approximately 40% bound protein Fv, whereas only a small percentage ($<$20%) of IgM from other V_H families bound protein Fv [23]. Inhibition studies suggested that all binding occurred at the same site(s) on protein Fv with very high affinity. They also investigated binding within a large antibody repertoire of a healthy adult obtained from combinatorial Fab phage display libraries. The results demonstrated that the affinity of protein Fv binding is comparable to that of a conventional antigen binding interaction, but the frequency of binders and the structural correlates for the interaction are akin to those of an immunoglobulin superantigen. This human liver protein was found to have the broadest of all known variable-region-mediated Fab-binding capacities and thus the highest frequency of binding interaction with the antibody repertoire of any protein characterized to date. Thus, within the gut, the compartmentalized and regulated expression of protein Fv may contribute to B-cell/antibody-mediated immune defense.

The binding site of protein Fv with human immunoglobulin V_H3+ was characterized by Silverman et al. [22]. They demonstrated that the superantigen properties of protein Fv and those of the bacterial protein A involve binding interactions with overlapping sites in the V_H3 domain. This indicated that self

(protein Fv) and foreign proteins (protein A) can use highly conserved strategies to create superantigens for the antigen receptors of B lymphocytes.

There is some indirect evidence that protein Fv can act as a human B-cell superantigen. It has been reported that protein Fv can suppress the pokeweed mitogen-induced proliferation of human B-cells [4]. Interestingly, more than 80% of B lymphocytes reside in or transit at some time through the mucosal immune system [9]. The discovery of an endogenous immunoglobulin superantigen (protein Fv) extended our understanding of the pathophysiological role of B-cell superantigens in lymphocyte activation and trafficking in the gut.

Superallergenic Properties of Protein Fv

The concept of immunoglobulin superantigens applied to the pathophysiology of allergic disorders could be translated as 'superallergens' so as to indicate proteins of various origins (self and foreign) able to activate mast cells and basophils through the interaction with membrane-bound IgE [24, 25].

Human basophils and mast cells are the only cells expressing the tetrameric high-affinity receptor of IgE (FcεRI) and synthesizing histamine [26]. Basophils and mast cells (FcεRI+ cells) play a prime role in the pathophysiology of allergic disorders through the elaboration and release of numerous proinflammatory and immunoregulatory molecules and the expression of a wide spectrum of receptors for cytokines and chemokines [27, 28].

There are four canonical mechanisms by which cross-linking the IgE-FcεRI network can induce the release of mediators from these cells [26, 28]. A multivalent antigen can cross-link at least two specific IgE molecules bound to FcεRI+ cells to release mediators [29]. Anti-human IgE antibodies (anti-IgE) possess two binding sites for the Fcε region of human IgE and are potent stimulators of histamine and cytokine secretion from these cells [30, 31]. Similarly, antibodies directed against epitopes of the α-chain of human FcεRI (anti-FcεRIα) can trigger the release of mediators from FcεRI+ cells [32]. Finally, it is possible that immune complexes containing IgG against human IgE can activate human basophils [33].

It is generally thought that these four canonical mechanisms of IgE-mediated activation of human FcεRI+ cells are responsible for the pathophysiological involvement of these cells in the majority of patients with allergic disorders [34]. However, there is evidence that a significant percentage of allergic diseases (e.g. certain cases of intrinsic asthma and chronic idiopathic urticaria) cannot be explained by the four classical mechanisms of FcεRI+ cell activation.

During recent years, we have investigated the possibility that alternative mechanisms of activation of human FcεRI+ could be involved in the pathogenesis of certain cases of allergic disorders. In particular, we examined the hypothesis that immunoglobulin superantigens of various origins (endogenous, bacterial and viral) can activate human basophils and mast cells to release proinflammatory mediators and cytokines.

Protein Fv Is an Endogenous Superallergen That Activates Human Basophils and Mast Cells

Acute and chronic viral hepatitis are associated with a myriad of extrahepatic tissue injuries, not all of which are directly related to the cytotoxic effects of HBV and HCV [35, 36]. These include skin rashes and urticarial reactions in about 5% of patients with viral hepatitis [37–40]. We investigated the possibility that protein Fv purified from patients suffering from HCV infection can activate basophils and mast cells purified from human lung and skin tissues. We found that protein Fv purified from stool extracts of patients with HCV infection induced histamine release from human basophils [32, 41]. Interestingly, the histamine-releasing activity of protein Fv was superimposible with its immunoglobulin-binding property (fig. 2).

When we compared the histamine-releasing activity of increasing concentrations of protein Fv from patients with HCV and anti-IgE on histamine release from human basophils and mast cells, we found that the concentration-response curves induced by protein Fv paralleled that induced by anti-IgE [32]. Protein Fv was approximately 100 times more potent than anti-IgE. The same preparations of protein Fv absorbed with protein A-Sepharose coated with human polyclonal IgG, which were negative for immunoglobulin binding, did not induce histamine release [32, 41]. Consequently, protein Fv was responsible for secretion of the mediator from human FcεRI+ cells, presumably by binding to immunoglobulins. Protein Fv acted as complete secretagogue because it also caused the de novo synthesis of both prostaglandin D_2 (PGD_2) and cysteinyl leukotriene C_4 (LTC_4) by lung mast cells [41].

IgE Are Involved in the Activating Property of Protein Fv

The relationship between protein Fv and IgE was investigated by performing cross-desensitization between these two stimuli [32, 41]. Human basophils and mast cells preincubated with protein Fv released approximately 90% less histamine when challenged with anti-IgE. Similarly, when protein Fv-pretreated

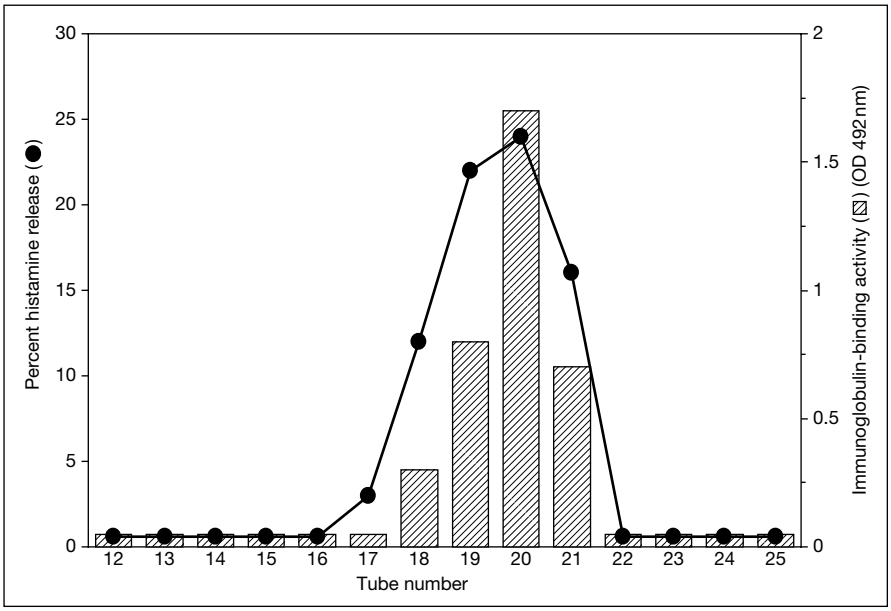

Fig. 2. High performance liquid chromatography fractions of stool extracts obtained from a patient with HCV were examined for their immunoglobulin-binding activity and histamine-releasing activity from human basophils. The dotted line represents the percent histamine release induced by the different fractions with basophils from a single donor. The vertical bars represent immunoglobulin-binding activity (OD 492 nm).

cells were challenged with anti-IgE, they lost their ability to release with the heterologous stimulus. These results are compatible with the hypothesis that the releasing property of protein Fv is mediated principally by interaction with IgE present on the basophil or mast cell surface. More direct evidence that protein Fv activates FcεRI+ cells by binding to IgE comes from the finding that protein Fv does not induce mediator release from basophils or mast cells stripped of IgE from FcεRI by brief exposure to lactic acid [32, 41].

Protein Fv Induces Mediator Release from FcεRI+ Cells through the Interaction with IgE V_H3+

To assess the mechanism by which protein Fv activates FcεRI+ cells, the protein was preincubated with human monoclonal IgM of different V_H families

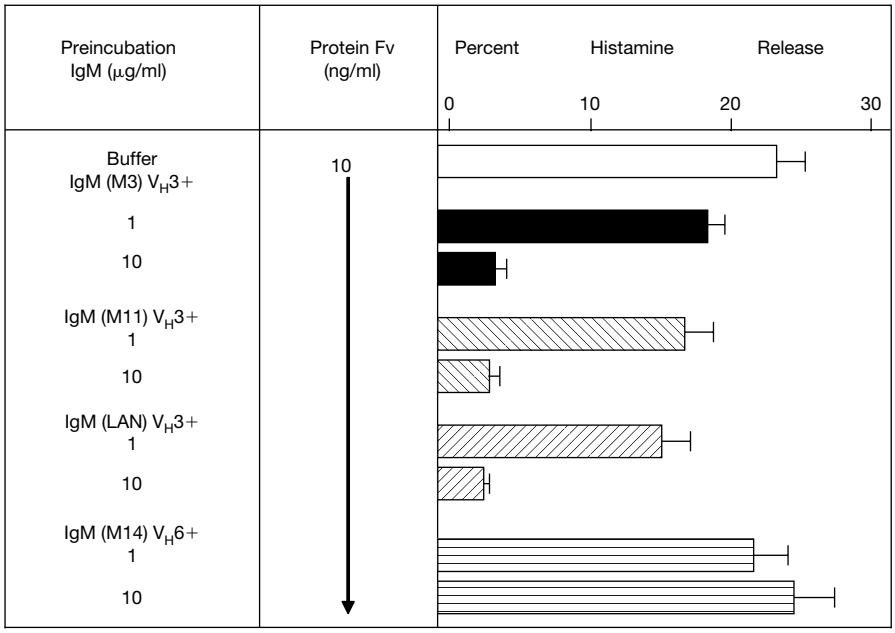

Fig. 3. Effects of preincubation of protein Fv with monoclonal IgM V_H3+ or IgM V_H6+ on histamine release from human lung mast cells. Protein Fv (10 ng/ml) was preincubated for 10 min at 37°C with increasing concentrations (1–10 μg/ml) of human monoclonal IgM V_H3+ (M3, M11, and LAN) or IgM V_H6+ (M14). Lung mast cells were then added, and incubation was continued for another 30 min at 37°C. Each bar shows the mean ± SEM of triplicate determinations.

[32, 41]. Preincubation of basophils with three preparations of IgM V_H3+ concentration-dependently inhibited the histamine-releasing activity of protein Fv. By contrast, preincubation with a monoclonal IgM which has a V_H6 domain had no such effect [32, 41]. Similar results were obtained when human lung mast cells were preincubated with three different preparations of human monoclonal IgM V_H3+ or IgM V_H6+ and then challenged with protein Fv (fig. 3). These results are compatible with the hypothesis that protein Fv binds to IgE V_H3+ bound to FcεRI+ cells.

Endogenous Superallergen Protein Fv Induces IL-4 from Human Basophils

We also demonstrated that low concentrations of protein Fv can induce IL-4 secretion from basophils [32]. Protein Fv induced an increase in IL-4 mRNA

similar to anti-IgE. The stimulation of IL-4 synthesis in basophils by protein Fv was mediated by interaction with IgE. In fact, brief exposure to lactic acid completely blocked the effect of protein Fv and of anti-IgE on IL-4 secretion from basophils. Preincubation of basophils with preparations of human monoclonal IgM V_H3+ [32] inhibited the IL-4-releasing activity of protein Fv. These results confirm that protein Fv activates human basophils by interacting with IgE V_H3+.

These results demonstrate that an endogenous superallergen, protein Fv, released during viral infections, can induce the synthesis and secretion of such an important cytokine as IL-4 from basophils. Interestingly, the in vitro activity of protein Fv is extremely potent, which raises the possibility that it exerts important effects in vivo. It is not inconceivable that protein Fv released in the blood of patients with acute and chronic HCV, HAV or HBV infection contributes, through the release of mediators from FcεRI+, to the allergic manifestations observed in some of these patients [37–40]. The activity of IL-4 has been linked to several immunoregulatory functions including the synthesis of IgE by B lymphocytes [42] and it is intriguing that some patients with viral hepatitis have high serum IgE levels [43, 44].

In conclusion, protein Fv, an endogenous protein present in normal liver and released in biological fluids during viral hepatitis [1], is a potent inducer of the release of proinflammatory mediators and cytokines from human FcεRI+. This was the first evidence that a human protein induced by viral infections can act as an endogenous immunoglobulin superantigen.

Protein Fv Is an Endogenous Superallergen That Activates Human Heart Mast Cells

The effects induced by viral hepatitis on the cardiovascular system are not widely recognized [45–48] and the association has probably been overlooked both clinically and pathologically. For instance, type II cryoglobulinemia, a systemic vasculitis caused by HCV infection [49, 50], can be associated with cardiovascular involvement [51]. The mechanism(s) by which viral infections impinge on these disorders is/are still largely obscure.

Mast cells are found in the human heart [31, 52], around coronary arteries [53], and in the coronary intima [54]. Human heart mast cells have been implicated in the pathophysiology of myocarditis [55, 56], coronary artery diseases [53] and dilated cardiomyopathy [57]. Finally, mast cells, through the release of vasoactive mediators, are also involved in systemic vasculitis [39].

We evaluated the effects of increasing concentrations of protein Fv obtained from patients suffering from HCV on the activation of human cardiac mast cells [58]. Low concentrations of protein Fv concentration-dependently

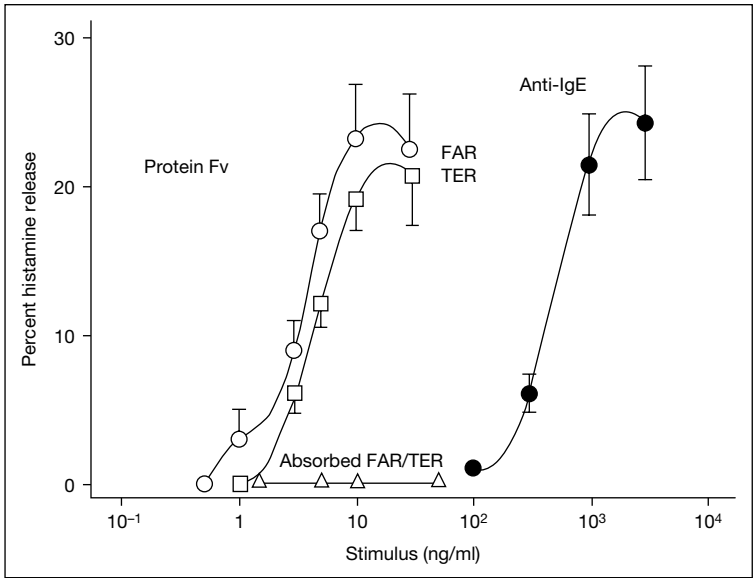

Fig. 4. Effects of increasing concentrations of proteins Fv and anti-IgE on histamine release from human cardiac mast cells from seven donors. Protein Fv from donors FAR and TER was absorbed or nonabsorbed with protein A-Sepharose coated with polyclonal IgG. Cells were challenged with protein Fv or anti-IgE for 30 min at 37°C. Values are expressed as mean ± SEM. Error bars are not shown when graphically too small. (Reproduced with permission from Genovese et al. [58].)

released a significant percent of histamine from cardiac mast cells isolated from six donors (fig. 4). The concentration-response curves induced by the preparations of protein Fv paralleled that induced by anti-IgE. However, protein Fv was approximately 100 times more potent than anti-IgE. The same preparations of protein Fv absorbed with protein A-Sepharose coated with human polyclonal IgG, which were negative for immunoglobulin binding, did not induce mediator release. We also found a significant correlation between the maximum percent histamine release induced by protein Fv and that induced by anti-IgE suggesting that protein Fv might induce histamine release from cardiac mast cells by interacting with IgE. Consequently, protein Fv was responsible for the mediator secretion from cardiac mast cells, presumably by binding to IgE.

Secretory granules of cardiac mast cells contain tryptase as well as histamine [31]. We found a significant correlation between the maximum histamine and tryptase release indicating that protein Fv releases tryptase in parallel with

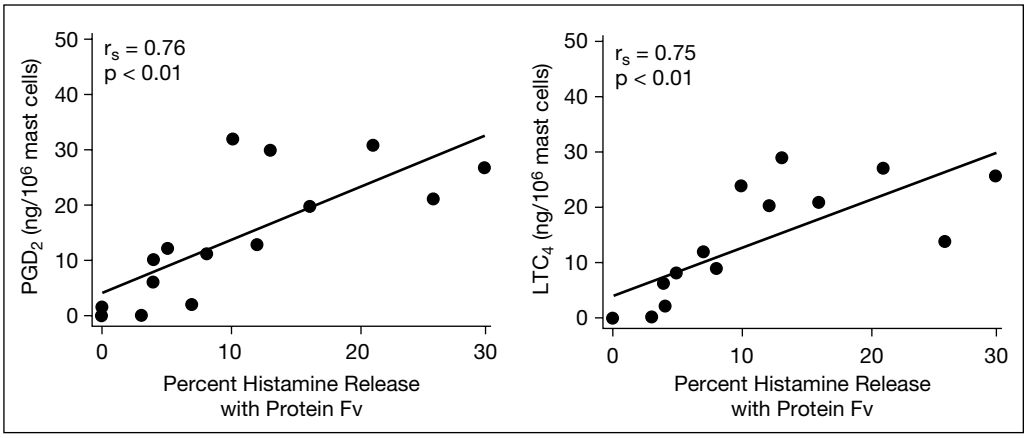

Fig. 5. Correlations between the maximum percent of histamine secretion and PGD_2 and LTC_4 release induced by protein Fv from human heart mast cells. Each point is the mean of duplicate determinations from separate experiments. (Reproduced with permission from Genovese et al. [58].)

histamine from cardiac mast cells. Human heart mast cells challenged with anti-IgE synthesize de novo PGD_2 and LTC_4 [31, 56], two proinflammatory mediators endowed with vasoactive and biological properties [59–62]. Protein Fv acted as complete secretagogue because it also caused the de novo synthesis of both PGD_2 and LTC_4 by cardiac mast cells (fig. 5).

To assess the mechanism by which protein Fv activates cardiac mast cells, the protein was preincubated with human monoclonal IgM of different V_H families [63]. Preincubation of mast cells with preparations of monoclonal IgM that possess a V_H3 domain concentration-dependently inhibited the releasing activity of protein Fv. By contrast, preincubation with a monoclonal IgM V_H6+ had no such effect. These results suggest that binding to the V_H3 domain inhibits the binding of protein Fv to IgE bound to FcεRI on cardiac mast cells.

Our results provided the first evidence that an endogenous superantigen, protein Fv, released during viral infections, can induce the in vitro synthesis and secretion of several important mediators from human heart mast cells. The activity of protein Fv is extremely potent, raising the possibility that it exerts important effects in vivo. Although as yet there are no data about serum concentrations of protein Fv in different pathophysiological conditions, it is not inconceivable that this protein synthesized by the liver in patients with acute and chronic HCV, HAV or HBV infection contributes, through the release of

mediators from FcεRI+, to some of the cardiovascular manifestations observed in some of these patients [45–48].

Given the biological importance of mast cell-derived mediators such as histamine [26, 64], tryptase [65], PGD_2 [59, 62], and cysteinyl leukotrienes [60, 61] in heart pathophysiology, our findings might explain the association between hepatitis viruses and cardiovascular damage in some patients with these infections. Interestingly, tryptase, a neutral protease present in the cytoplasmic granules of cardiac mast cells [31, 56], activates complement, thereby leading to the formation of anaphylatoxins (C3a and C5a) [65]. C5a receptors are present on cardiac mast cells and their engagement by C5a leads to their activation and the release of proinflammatory mediators [31].

Protein Fv activation of cardiac mast cells represents a new model for a pathogenetic link between viral hepatitis infections and FcεRI+ cell activation. More generally, the mechanism of FcεRI+ cell activation by protein Fv represents a new pathogenetic cascade involving viral infection, endogenous immunoglobulin superantigen production, activation of human cardiac mast cells and tissue injury. This raises the possibility that additional viruses can induce the release of an endogenous immunoglobulin superantigen (e.g. protein Fv) which can cause tissue injury through this mechanism that involves activation of cardiac mast cells.

Closing Thoughts

There is compelling evidence that viral (gp120) and bacterial proteins (protein A of *S. aureus* and protein L of *P. magnus*) can act as immunoglobulin superantigens. Protein Fv is the prototype of an endogenous mammalian protein acting as an immunoglobulin superantigen. Bouvet et al. [1, 3] purified this liver protein from stool extracts obtained from patients with viral hepatitis. They characterized the immunoglobulin-binding activity of protein Fv by demonstrating that each molecule of this protein possesses at least six binding sites for the V_H region of human immunoglobulin isotypes. Silverman et al. [22, 23] investigated the molecular basis for antibody binding of protein Fv and they demonstrated that protein Fv binds to human immunoglobulin V_H3+ with high affinity.

What is the physiological role of this liver protein? Bouvet et al. [6] suggested that, in physiological conditions, protein Fv synthesized by the liver can be released through the bile into the gut. Within the gut, protein Fv binds to sIgA thereby favoring the polymeric status of immunoglobulins. The formation of high-molecular-weight complexes can amplify the agglutination property of sIgA. Thus, protein Fv could play a role in the immune defense in the gut.

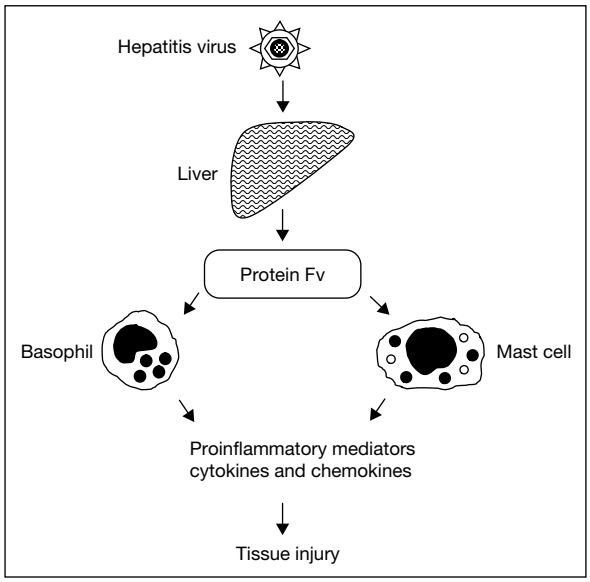

Fig. 6. A new pathogenetic link between viral infection and tissue injury. Protein Fv, synthesized in low amounts in normal liver, is released in the biological fluids of patients affected by acute and chronic viral hepatitis. Protein Fv has six binding sites for the V_H3 region of human immunoglobulin and is a potent stimulator of histamine and cytokine release from FcεRI+ cells through the interaction with V_H3+ IgE. This illustrates a novel mechanism by which an endogenous protein (protein Fv) activates human FcεRI+ cells, thereby acting as immunoglobulin superantigens.

Bouvet and colleagues [32, 41, 59, 63, 66] were able to characterize the immunoglobulin-binding properties of protein Fv in great detail. However, the superantigenic activity of the protein remained less well defined partly because of the limited quantity of purified protein Fv available for such studies. In collaboration with Bouvet, we extensively investigated the superallergenic properties of protein Fv. We found that different preparations of protein Fv activate human basophils and mast cells isolated from different tissues to release proinflammatory mediators and cytokines. Protein Fv acts as an endogenous superallergen by interacting with IgE V_H3+ to induce the release of mediators from human FcεRI+ cells.

The mechanism of FcεRI+ cell activation by protein Fv represents a new pathogenetic cascade consisting of viral infection, endogenous immunoglobulin superantigen production, activation of human basophils/mast cells and tissue injury (fig. 6). These observations raise the possibility that unconventional endogenous superallergens play a pathophysiological role in certain forms of allergic disorders.

Also of interest is the observation that protein Fv is a potent inducer of mediator release from mast cells isolated from human heart tissue [58, 63]. This represents the first evidence that a human protein induced by viral infections can act as an endogenous immunoglobulin superantigen causing the release of vasoactive and immunoregulatory mediators from cardiac mast cells.

Although there are no data about the serum concentrations of protein Fv in different pathophysiological conditions, it is not inconceivable that this protein synthesized by the liver in patients with acute and chronic viral hepatitis infections can contribute, through the release of mediators from cardiac mast cells, to some of the cardiovascular manifestations observed in some of these patients [45–48].

HCV has been associated with many extrahepatic manifestations [35, 36, 67], including myocarditis and cardiomyopathy [68, 69]. Histological and functional observations have led to the hypothesis that mast cells play an important role in many fibrotic reactions [70–72]. Potential mast cell-derived mediators of the fibrotic response include histamine, tryptase and TGF-β_1, which stimulate fibroblast proliferation and collagen synthesis [73–75]. By producing these factors, chronic activation of cardiac mast cells by protein Fv may contribute to the heart fibrosis found in some patients with HCV infection [68, 69].

In conclusion, these findings emphasize the possibility that protein Fv is a relevant molecule in vivo during certain viral infections. It is evident that protein Fv is a fascinating protein that possesses several important immunological and biochemical properties. Unfortunately, this molecule is difficult to purify to homogeneity and has not yet been cloned. We should be extremely grateful to Jean-Pierre Bouvet who identified and purified this protein and generously made it available to many laboratories. It has been a real pleasure and a great honor for me to collaborate with this gentleman scientist whose premature death has prevented a complete characterization of this fascinating molecule.

Acknowledgements

This work was supported by grants from the Istituto Superiore Sanità (AIDS Project 40.C.60 and 40.D.57), Ministero della Salute 'Alzheimer Project', Ministero dell'Università e della Ricerca (MUR) (Rome, Italy) and Regione Campania (Naples, Italy).

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Marone G (ed): Superantigens and Superallergens.
Chem Immunol Allergy. Basel, Karger, 2007, vol 93, pp 77–91

Yersinia pseudotuberculosis Superantigens

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Abstract

Yersinia pseudotuberculosis, a gastro-intestinal bacterium, produces three closely related T cell superantigens, YPMa, YPMb and YPMc, which have no significant sequence similarity to other proteins, let alone other bacterial superantigens. *Y. pseudotuberculosis*-derived mitogen (YPM) has been shown to play a role in the pathogenesis of human and animal *Y. pseudotuberculosis* infection. The three-dimensional structure of YPMa, as determined by X-ray crystallography and nuclear magnetic resonance spectroscopy, exhibits a jelly roll fold, a structural motif not observed in other superantigens. YPMa is structurally most similar to virus capsid proteins and members of the tumour necrosis factor (TNF) superfamily. In the crystal structure, YPMa forms a trimer, another feature shared with virus capsid proteins and TNF superfamily proteins. However, in solution YPMa exists as a monomer, and any functional relevance of the trimer observed in the crystals is yet to be established. Structures of YPM bound to the T cell receptor and/or the major histocompatibility complex (MHC) are not yet available and mapping of existing mutagenesis data onto the three-dimensional structure of YPMa did not reveal potential T cell receptor/MHC binding sites. Knowledge of the structure will aid the design of functional studies aimed at further characterizing this superantigen.

Pathogenic Yersinia Species

Yersinia pseudotuberculosis is a Gram-negative gastro-intestinal pathogen belonging to the Enterobacteriaceae family. The *Yersinia* genus contains 11 identified species, 3 of which are known human pathogens: *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis*. Although *Y. enterocolitica* and *Y. pseudotuberculosis* are both enteropathogens and display relatively similar infection symptoms, they are distantly related. In contrast, *Y. pseudotuberculosis* and *Y. pestis*, the causative agent of plague, are genetically similar and population

genetic studies suggest that *Y. pestis* evolved from *Y. pseudotuberculosis* 1,500–20,000 years ago, shortly before the first known pandemics of human plague [1]. Comparison of *Y. pseudotuberculosis* and *Y. pestis* whole genome sequences has shown that extensive gene inactivation and insertion sequence-mediated genome rearrangements were major contributors to the evolution of such a highly virulent clone from a less virulent ancestor with markedly different ecology and clinical manifestations [2, 3]. It was estimated that up to 13% of *Y. pseudotuberculosis* genes no longer function in *Y. pestis*, while 32 *Y. pestis* chromosomal genes and 2 *Y. pestis*-specific plasmids are the only new genetic material in *Y. pestis* acquired since its divergence from *Y. pseudotuberculosis* [2]. T cell superantigens identified in *Y. pseudotuberculosis* play a role in the pathogenesis of *Y. pseudotuberculosis* infections. Before discussing *Y. pseudotuberculosis* superantigens in detail, some general information on *Y. pseudotuberculosis* infection and virulence factors is provided in the following sections.

Y. pseudotuberculosis Infection

A detailed review of *Y. pseudotuberculosis* epidemiology has recently been published [4]. Environmental pools of *Y. pseudotuberculosis* are found in soil and water, and the bacterium has been isolated from a large variety of domestic and wild animals, with more widespread distribution in the northern hemisphere, particularly in countries with a temperate climate. Human outbreaks have mainly been reported in Japan, Finland, the former Soviet Union and Canada [5–10]. The mode of transmission of this pathogen has remained largely obscure, although numerous links of outbreaks have been made with contaminated water, contact with infected animals, and food [11–16]. Recent evidence suggests that *Y. pseudotuberculosis* is indeed a foodborne pathogen when iceberg lettuce was identified as the likely carrier of a *Y. pseudotuberculosis* outbreak in Finland [17].

The pathophysiology of *Y. pseudotuberculosis* infection involves colonization of the digestive tract, translocation through the gut epithelium, establishment within Peyer's patches, and transport to other organs [4]. Histopathological investigations have shown that, in contrast to *Y. enterocolitica,* acute enterocolitis and terminal ileitis are relatively rare during *Y. pseudotuberculosis* infections, which usually result in mesenteric adenitis without visible bacteria. *Y. pseudotuberculosis* infection is characteristically associated with a strong macrophage reaction in the paracortical area of mesenteric lymph nodes, sometimes in the form of epithelioid granulomas of the tuberculoid type (from which the species name was coined).

Infection with *Y. pseudotuberculosis* is associated with a wide variety of clinical symptoms including fever, rash, abdominal pain, vomiting, diarrhoea

and arthritis. For example, an early study of 25 patients with intestinal yersiniosis reported that *Y. pseudotuberculosis* (15 patients) and *Y. enterocolitica* (10 patients) caused identical symptoms: fever (in 80% of patients), abdominal pains (56%), diarrhoea (52%), erythema nodosum (44%), arthritis (40%) and vomiting (16%) [18]. The initial symptoms of gastro-enteritis and fever were usually followed by a second phase with returning fever, abdominal pains, erythema nodosum and/or arthritis. The mechanisms by which *Y. pseudotuberculosis* induces and/or mediates these symptoms are poorly understood, however *Y. pseudotuberculosis* superantigens may play a pathogenic role by inducing excess immune stimulation in the classical superantigen manner.

Y. pseudotuberculosis has also been implicated in Kawasaki disease, an acute vascular syndrome that is the leading cause of acquired heart disease in infants and children in developed countries [19–22]. With regard to the aetiology of Kawasaki disease, it is generally agreed that an infectious trigger initiates hyperstimulation of the immune system resulting in a prolonged auto-immune response, predominantly targeting the coronary arteries. However, Kawasaki disease remains poorly characterized and a definite causative link between *Y. pseudotuberculosis* infection and the disease has not yet been established. If Kawasaki disease is associated with *Y. pseudotuberculosis* infection then it is possible that *Y. pseudotuberculosis* superantigens play a role in immune activation of the infected host.

A rare septicaemic form of *Y. pseudotuberculosis* infection in humans has also been reported. A review of the literature found 54 cases of septicaemic infection which was usually observed in patients with underlying disorders such as diabetes, hepatic cirrhosis or iron overload [23]. Recently, HIV-related immunosuppression has been shown to be an additional potential risk factor for *Y. pseudotuberculosis* septicaemia [24].

Virulence Factors

In common with the other pathogenic yersiniae, virulence of *Y. pseudotuberculosis* is dependent on the presence of a 70-kb virulence plasmid pYV (plasmid associated with *Yersinia* virulence). Encoded in pYV is a type III secretion system which is necessary for survival and replicates within lymphoid tissues of animal or human hosts [25]. Also encoded in the pYV plasmid are a set of pathogenicity factors, including those known as Yersinia outer proteins. Yersinia outer proteins are exported by the type III secretion system upon bacterial infection of host cells, and function to disrupt innate and adaptive immune responses to infection [26].

Two virulence factors involved in *Y. pseudotuberculosis* and *Y. enterocolitica* pathogenicity are adhesion factors which are not active in *Y. pestis.* YadA is

encoded in the pYV virulence plasmid and allows binding and internalization of host cells, while invasin, a chromosome-encoded adhesion molecule, binds β_1 integrins on the surface of epithelial cells [27].

Pathogenic yersiniae are subdivided into low- and high-pathogenicity strains. Low-pathogenicity strains cause mild gastro-intestinal symptoms in humans and are not lethal to mice at low doses, whereas high-pathogenicity strains are associated with more severe systemic infections in humans and are lethal to mice at low doses [28]. A high-pathogenicity island (HPI), which encodes for a yersiniabactin system responsible for iron uptake, is present only in highly pathogenic strains of all three yersiniae [29]. Horizontal transfer of HPI between certain strains of *Y. pseudotuberculosis*, from *Y. pseudotuberculosis* to *Y. pestis*, but not from *Y. pseudotuberculosis* to *Y. enterocolitica,* has been demonstrated [30]. The fact that this transfer occurred specifically in conditions of low temperature and iron-limited media implies the natural environment of the bacterium may be a positive selection condition for the lateral transfer of HPI [30].

An additional pathogenicity island, YAPI (*Yersinia* adhesion pathogenicity island), has recently been characterized in *Y. pseudotuberculosis* [31]. The 98-kb chromosome fragment includes a type IV pilus gene cluster and a large number of mobile genetic elements. A homologue of this pathogenicity island was detected in *Y. enterocolitica* but was not present in *Y. pestis.*

The T cell superantigens from *Y. pseudotuberculosis* can be considered virulence factors, as discussed below. Limited reports that *Y. enterocolitica* produces a superantigenic substance which is able to stimulate human and murine T cells in a Vβ- and major histocompatibility complex (MHC) class II-dependent manner have been published [32, 33]. The *Y. enterocolitica* superantigen has not been well characterized, however the mitogen stimulates variable elements, i.e. Vβ3, Vβ12, Vβ14 and Vβ17 on human T cell receptors (TCRs). To date, there are no reported superantigens produced by *Y. pestis.*

Y. pseudotuberculosis Superantigens

Y. pseudotuberculosis produces superantigens termed *Y. pseudotuberculosis*-derived mitogens (YPMs). YPM was first isolated from patients with *Y. pseudotuberculosis* infection and was recognized as a T cell stimulant in 1993 [34–36]. YPM, at concentrations as low as 0.1 ng/ml, was shown to stimulate human peripheral T cells to produce interleukin-2. YPM selectively stimulates human T cells bearing TCRs with Vβ3, Vβ9, Vβ13.1 and Vβ13.2 elements, in an MHC class II-dependent manner without being processed by antigen-presenting cells. In mice, YPM stimulates T cells bearing TCRs with Vβ7, Vβ8.1, Vβ8.2 and

Vβ8.3 elements [37]. A pathogenic role for YPM has been established in a number of studies and in vivo production of YPM has been detected in clinical patients infected with *Y. pseudotuberculosis.* Infection was accompanied by an IgG response and elevated levels of Vβ3 T cells in peripheral blood mononuclear cells and mesenteric lymph nodes [38]. In mice, YPM has been shown to induce an acute, T-cell-mediated, toxic shock response [39]. The toxic shock response was inducible in BALB/c mice but not in T-cell-deficient SCID mice. As early as 1 h after YPM injection, Vβ8+ T cells were observed in the liver while serum levels of interferon-γ were significantly elevated 4 h after injection. Co-administration of anti-interferon-γ antibody or anti-YPM monoclonal antibody alleviated the liver injury and protected mice from YPM-induced death. Anti-YPM antibody also suppressed the early migration of Vβ8+ T cells from the peripheral circulation and the elevation of serum interferon-γ.

A study using a *ypm*-deficient strain of *Y. pseudotuberculosis* showed that YPM contributes to the virulence of *Y. pseudotuberculosis* in systemic infection. Mice inoculated with this strain showed a higher survival rate compared to mice inoculated with the wild-type strain [40]. OF1 mice inoculated intravenously with 10^4 and 10^5 wild-type bacteria died within 9 days, whereas mice infected with the *ypm*-deficient strain survived 12 and 3 days longer, respectively. The decreased virulence of the *ypm*-deficient strain was not due to an impaired bacterial colonization of the spleen, liver or lungs of infected animals. In contrast to intravenous challenge, bacterial inoculation by the oral route did not reveal any difference in virulence between wild-type *Y. pseudotuberculosis* and the *ypm*-deficient strain since the 50% lethal doses (LD_{50}) were identical for both strains. Moreover, inactivation of the *ypm* gene did not affect the bacterial growth of *Y. pseudotuberculosis* in Peyer's patches, mesenteric lymph nodes and spleen after oral inoculation. Histological examination of the spleen, liver, lungs, heart, Peyer's patches, and mesenteric lymph nodes after intravenous or oral challenge with the wild-type or the *ypm*-deficient strain did not reveal any feature that could be specifically attributable to YPM.

YPM-mediated immune activation may play a role in the pathophysiology of gut dysfunction observed during *Y. pseudotuberculosis* infection [41]. In vitro, YPM was able to alter epithelial function by reducing active ion transport and increasing epithelial permeability. Interferon-γ, interleukin-2, and tumour necrosis factor (TNF)-α were implicated in these processes. Colonic tissue from YPM-treated mice (20 μg intraperitoneally) displayed reduced responsiveness to cAMP-mediated secretagogues and nerve stimulation. Immune activation by YPM in vivo was demonstrated by increased spleen cell proliferation and production of interleukin-2.

YPM has no amino acid sequence similarity to any other protein and its molecular weight (14 kDa) is much lower than that of the staphylococcal and

streptococcal superantigens (22–29 kDa). Three variants of YPM have been characterized to date: YPMa, YPMb and YPMc, encoded by *ypmA*, *ypmB* and *ypmC* genes, respectively. The YPM variants are highly homologous: YPMb has 83% sequence identity to YPMa, with the central region having the greatest variation [42]. The T cell stimulation profile for YPMb is the same as that of YPMa, however YPMb has not been detected in clinical strains of *Y. pseudotuberculosis* [43]. YPMc differs from YPMa only at position 51 where a histidine is substituted by a tyrosine residue in YPMc.

As indicated above, genes of the three YPM variants are not found in all *Y. pseudotuberculosis* strains. There is a clear geographical distribution pattern among clinical strains of *Y. pseudotuberculosis.* In one study, the *ypmA* gene was present in 100% of clinical strains isolated in Eastern Russia, 95% of clinical isolates from Japan, and 17% of European clinical strains [44]. A detailed study involving the analysis of 2,235 strains of *Y. pseudotuberculosis* for the presence of *ypm*, pYV and HPI genes was performed to assess patterns of geographical distribution and pathogenicity, as well as to investigate possible evolutionary paths of the organism [43]. Strains were classified into six groups. The majority of the clinical strains belonged to two groups showing clear geographical patterns: the YPM− HPI+ group which included European gastro-enteric pathogenic strains, and the Far Eastern systemic-pathogenic types which were YPMa+ HPI−. A low-pathogenicity European group was classified which was YPMc+ but only contained the right-hand side of the HPI. The right-hand side of the HPI is highly conserved in all three pathogenic yersiniae [28]. Non-pathogenic strains of *Y. pseudotuberculosis* constituted a separate group and were YPMb+ HPI−. Two separate groups which contained clinical strains and were pathogenic were the YPMa+ HPI+ group, and the YPM− HPI− strains.

YPM genes have a low GC content, 35% for YPMa and YPMb, compared with 47% for the *Y. pseudotuberculosis* genome. It was proposed that *Y. pseudotuberculosis* may have obtained *ypm* from a micro-organism with a low GC content, and the superantigen-producing species, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Mycoplasma arthritidis*, were suggested as candidates. However, significant differences in codon usage suggest that *ypm* was not acquired from these species [45, 46]. Unlike many staphylococcal and streptococcal superantigen genes which are found on phage DNA, plasmid DNA and pathogenicity islands, *ypm* genes are not associated with mobile genetic elements. They are instead located in an unstable locus of the genome between ORF3 and ORF4 [46]. It was speculated that a bacteriophage was involved in the incorporation of *ypm* into *Y. pseudotuberculosis.* A putative phage integration site is found 245 bp upstream of the *ypm* genes, however no phage remnants were detected in the vicinity of this nucleotide motif [46]. Recent evidence has indeed shown that the *ypm* gene has been horizontally acquired by

Y. pseudotuberculosis [47]. The *pil* gene cluster which encodes for the type IV pilus of YAPI is also laterally acquired in *Y. pseudotuberculosis*, and this has a significant association with *ypm* [47]. A type IV pilus may function as a bacteriophage receptor. Given that both *Y. pseudotuberculosis* and *Y. enterocolitica* possess *pil* gene clusters and YAPI homologues, but *Y. enterocolitica* does not harbour the *ypm* gene, this would imply that the *ypm* gene was acquired by *Y. pseudotuberculosis* after the divergence from a common *Yersinia* ancestor.

Structure of *Y. pseudotuberculosis*-Derived Mitogen

The best characterized superantigens are those from the Gram-positive pathogens *S. aureus* and *S. pyogenes.* The three-dimensional structures of these superantigens are closely related and consist of two distinct domains: an N-terminal domain similar to the oligosaccharide/oligonucleotide-binding fold, and a C-terminal domain similar to the β-grasp fold. The binding site for the TCR β-chain is located in a cleft between the two domains [48, 49]. We have recently published the crystal and solution structures of YPMa expressed as a recombinant protein in *Escherichia coli* [50, 51]. The structure is unlike that of any known superantigen and consists of a single domain which adopts a jelly roll fold made up of two β-sheets, each of which contains four antiparallel β-strands. β-Sheet 1 contains strands A, H, C and F, whilst β-sheet 2 contains strands B, G, D and E (fig. 1). Part of the polypeptide at the C-terminus runs transverse to β-sheet 2 and contains one turn of a 3_{10}-helix followed by two backbone-to-backbone hydrogen bonds with β-strand B. A disulphide bond is present between the two cysteines at position 32 and 129, and its disruption results in loss of activity of the protein [52]. The N-terminus extends away from the body of the structure and the first residue visible in electron density is isoleucine 15. The approximate dimensions of the molecule are $45 \times 30 \times 30$ Å. The solution structure obtained by nuclear magnetic resonance (NMR) spectroscopy is in overall good agreement with the crystal structure, displaying the same backbone fold. The most significant differences between the crystal and solution structures are seen in the interstrand loops connecting strands B and C, D and E, and F and G, as well as the C-terminus. These consist of the regions of the molecule which are most flexible, and that are undergoing conformational exchange in solution [51]. The coordinates of the crystal and NMR structures of YPMa are available from the Protein Data Bank (www.rcsb.org) under accession codes 1PM4 and 1POQ, respectively.

YPMa is structurally most similar to virus capsid proteins and members of the TNF superfamily (table 1). However, there is no apparent amino acid sequence basis for the observed structural similarities. Structure-based

Fig. 1. Ribbon diagram of the X-ray crystal structure of YPMa. The N- and C-termini of the polypeptide are labelled in black text while the β-strands are labelled in white text.

sequence alignments of YPMa with virus capsid proteins and members of the TNF superfamily resulted in sequence identities of only 4–13% for the 10 most closely related structures with no pattern of conserved residues. Moreover, structures with very low similarities to YPMa have sequence identities as high as, and in some cases higher than, the closest structural relatives. YPMa shares another structural feature with virus capsid proteins and TNF superfamily members. In the crystal structure, YPMa packs as a trimer with the three YPMa molecules related by 3-fold rotational symmetry, whereas members of the TNF superfamily function as trimeric proteins and virus capsid proteins form trimeric structures as part of icosahedral assemblies (fig. 2). The structural similarity between TNF-α and virus capsid proteins was first noted in 1989 and a potential evolutionary link was proposed [53, 54]. Such an evolutionary link received further support when the crystal structure of the B-cell-activating factor (BAFF), a TNF superfamily member, revealed a virus-like assembly of trimers containing 60 BAFF monomers [55]. This remarkable assembly of approximately 200 Å in diameter was detected in solution using gel filtration and light scattering and may be physiologically relevant [56].

Table 1. Protein structures most closely related to YPMa as determined by the Dali web server [63]

Protein	RMSD, Å	C^{α} atoms aligned	Percent sequence identity	Protein data bank code
Satellite tobacco necrosis virus	2.8	98	8	2STV
Cucumber mosaic virus	3.0	93	8	1F15
Adipocyte complement-related protein[1]	2.5	85	8	1C28
CD40 ligand fragment[1]	2.9	89	13	1ALY
Satellite panicum mosaic virus	3.0	90	6	1STM
Cowpea chlorotic mottle virus	2.9	90	11	1CWP
Tobacco ringspot virus	3.2	93	13	1A6C
Satellite tobacco mosaic virus	2.5	81	6	1A34
Cricket paralysis virus (vp1)	2.7	94	11	1B35
Adenovirus type 2 hexon	3.4	98	4	1DHX
TRAIL[1]	2.9	89	7	1D4V
Human rhinovirus 16 (vp1)	2.8	93	8	1AYM

RMSD = Root mean square deviation; TRAIL = TNF-related apoptosis-inducing ligand.
[1]Member of the TNF superfamily.

In solution, however, YPMa exists as a monomer as shown by gel filtration chromatography, analytical ultracentrifugation, and NMR experiments [51]. There are no structures available of YPM in complex with either MHC or TCR molecules, and functional studies to test whether the trimeric formation observed in the crystal structure is physiologically significant are lacking. We speculated that a YPM trimer may be functionally relevant and that such an assembly may form only upon interaction with the MHC and TCR in the molecularly crowded cell-cell interface that constitutes the immunological synapse [51, 57]. The surface buried in the trimer is sufficiently large in area to suggest that the interaction may be immunologically relevant. The mean buried surface area between the three monomer-monomer interfaces in the trimer is 960 $Å^2$ which is much smaller than the values observed for TNF superfamily members (e.g. TNF-α: 2,350 $Å^2$, BAFF: 1,870 $Å^2$). However, the buried surface area observed for one of the virus capsid trimer interfaces, that for cucumber mosaic virus, is only 620 $Å^2$, i.e. 35% smaller than that of YPMa. In addition, the buried surface area of 960 $Å^2$ calculated from the YPMa trimer corresponds to only a 10% probability of being a non-specific interaction according to an empirical analysis of protein-protein interactions observed in crystals [58]. Thus, the observed buried surface area in the YPMa trimer, along with the observation that virus capsids can possess smaller buried surfaces in their trimer interfaces,

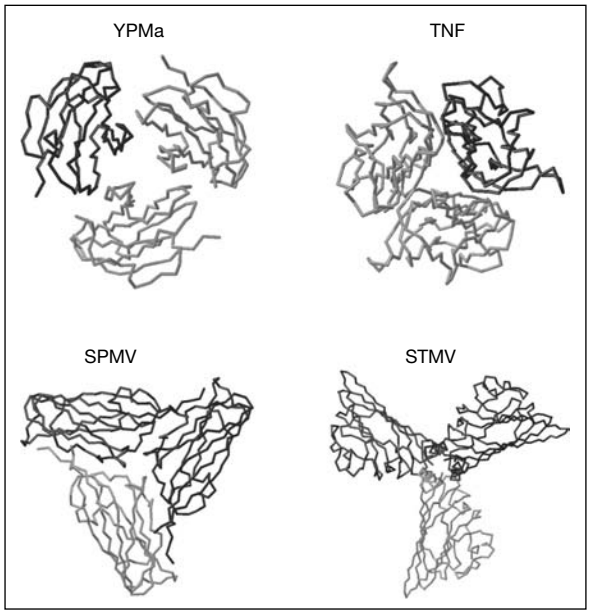

Fig. 2. The YPMa trimer shown with trimeric structures of virus capsid proteins and members of the TNF superfamily highlights the diversity of trimerization of jelly roll folds. Only C^{α} atoms of the polypeptide chains are shown. Virus trimers were extracted from the Viper database of virus structures (http://viperdb.scripps.edu/) [64]. SPMV = Satellite panicum mosaic virus; STMV = satellite tobacco mosaic virus. Figures 1 and 2 were created using Swiss-PdbViewer [65].

indicate that trimerization may be functionally important. Multimerization may play a role in the function of other superantigens. In particular, dimerization has been proposed to be functionally important for some staphylococcal and streptococcal superantigens. In the case of staphylococcal enterotoxin D and streptococcal pyrogenic exotoxin C, dimer formation was proposed as a means of cross-linking pairs of TCR and MHC molecules at the cell-cell interface [59, 60]. The YPMa trimer interface is formed predominantly from strand F residues 85–92 of one molecule and strand E residues 70–77 of a neighbouring molecule. This packing of monomers in the trimer differs from that observed for TNF superfamily members. For example, in TNF-α interchain interactions occur between all β-strands in sheet 1 of one molecule and β-strands D, E, F of a neighbouring molecule. This difference in packing results in the DE loop residing on the exterior of the trimer while for YPMa the DE loop is situated near the intermolecular interface of the trimer. While TNF superfamily members share a

conserved mode of trimerization, virus capsid proteins exhibit variable trimer packing orientations as shown in figure 2.

The structural similarity of YPM with virus capsid proteins and TNF superfamily members raises the possibility of an evolutionary relationship between these proteins. As discussed above, some staphylococcal and streptococcal superantigens are encoded by bacteriophages; however, these superantigens are not structurally related to any known virus proteins. While no viral superantigens have been structurally characterized, the observed similarity of YPM with virus capsid proteins suggests a potential evolutionary link between viral and bacterial superantigens.

A number of functional studies aimed at characterizing the molecular interaction between YPM and the TCR and MHC class II molecule have been reported. In one study, seven overlapping synthetic peptides covering the entire YPMa molecule were tested for an effect on YPMa-induced proliferation of human lymphocytes. A peptide corresponding to residues 1–23 of YPMa was found as the best proliferation inhibitor causing 50% inhibition, suggesting competition of this peptide with YPMa for the TCR or the MHC [61]. However, when YPMa was expressed in *E. coli* for structure determination, residues 1–13 were prone to heterogeneous proteolysis, and these residues were not visible in the crystal structure [51]. The N-terminal region, up to residue 20, is also ill-defined in the NMR structure [51]. If the N-terminus is important for the binding of YPM, it may only adopt a stable structure when complexed to the TCR/MHC.

Mapping of existing mutagenesis data onto the three-dimensional structure of YPMa did not reveal potential binding sites for the TCR and/or MHC [51]. In an extensive study of site-directed mutants of YPMa, a number of mutants were produced with reduced T cell proliferative activity [52]. However, most of the low-activity mutants are buried in the structure and the reduced activity of these mutants may be a consequence of conformational changes or partial misfolding of mutant YPMa molecules. Only five of the low-activity mutants involve residues on the surface of the protein which are potentially capable of forming direct TCR or MHC contacts. However, these five residues are scattered over the surface of the molecule and are not suggestive of a possible binding surface. Similarly, the mapping of full-activity mutants onto the three-dimensional structure did not reveal any obvious contiguous surface not involved in TCR or MHC interactions.

In another study, six point mutants defective in their binding of the TCR Vβ region, and with corresponding diminished cytotoxic activation and reduced production of pro-inflammatory cytokines, were characterized [62]. However, as for the more extensive study, this study when combined with structural knowledge of YPMa does not provide additional insight into potential

binding sites. It is clear that more mutagenesis studies are required and that the design of these studies can be aided by knowledge of the structure of YPMa.

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Marone G (ed): Superantigens and Superallergens.
Chem Immunol Allergy. Basel, Karger, 2007, vol 93, pp 92–105

B Cell Superantigens Subvert Innate Functions of B Cells

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Abstract

Some infectious agents produce molecules capable of interacting specifically with the immunoglobulin variable regions, independently of the conventional binding site. They are referred to as B cell superantigens, and include protein A of *Staphylococcus aureus*, protein L of *Peptostreptococcus magnus*, and gp120 of HIV-1. To test their effects in vivo, we used transgenic mice whose immunoglobulin loci were inactivated and their humoral immune system had been restored by introduction of human immunoglobulin loci. We found that administration of soluble recombinant B cell superantigens can deplete B cell subsets in vivo. They also preferentially impact B cell subpopulations responsible for innate-like functions. The studies indicate that certain pathogens have evolved primarily to subvert innate functions of B cells.

An intriguing feature of some infectious agents is the propensity to produce microbial products able to interact with specific portions of immunoglobulins (Igs). For example, protein G from group C and D streptococci binds to the C_H2 and C_H3 domains of human γ heavy (H) chains, and *Staphylococcus aureus* protein A (SpA) binds to the C_H2 and C_H3 domains of human γ, μ and α H chains. It is even more remarkable that some infectious agents produce molecules capable of interacting specifically with the Ig H or light (L) chain variable regions, independently of the conventional binding site. They are referred to as B cell superantigens (SAgs) and include protein A of *S. aureus,* gp120 of HIV-1, and protein L of *Peptostreptococcus magnus* [1–4]. In this mode of interaction, the variable region determines nearly exclusively the specificity of the Ig for the B cell SAg. This unconventional type of B lymphocyte ligands can, in principle, trigger all B cells bearing the appropriate variable region, regardless

of the other junctional and diversity segments. While conventional antigens stimulate a small proportion of B cells, the proportion of B cells responsive to SAgs can be orders of magnitude higher. This unconventional mode of interaction with B cells, which is comparable to that of known SAgs with T lymphocytes [5], is being investigated for HIV-1 antigens, and a number of abnormalities and fluctuations of the B cell repertoire have been described [3, 6–12].

S. aureus Protein A Targets B-1a Cells and Marginal Zone B Cells

S. aureus is a leading cause of human infections worldwide. It causes a variety of diseases, including impetigo, cellulitis, food poisoning, toxic shock syndrome, necrotizing pneumonia, endocarditis and sepsis [13–15]. Risk of *S. aureus* is increased during hospitalization, and in patients with indwelling percutaneous medical devices and/or other catheters. Notably, there is an alarming increase in the incidence of community-acquired *S. aureus* infections in seemingly healthy individuals, a problem which underscores the need to better understand virulence mechanisms. A number of factors are known to contribute to the pathogenesis. However, a comprehensive analysis of changes in *S. aureus* gene expression that occur during interaction with the human innate immune system has not been performed. Furthermore, our understanding of the molecular mechanisms used by pathogens to avoid destruction by innate host defenses is limited.

Even though *S. aureus* produces a variety of virulence factors, protein A (SpA) is the prototype staphylococcal surface protein [15]. During infection, SpA has antiphagocytic properties that are based on its ability to bind the Fc portion of Igs. In addition to its Fc IgG binding capacity, which has been used for Ig purification, SpA also interacts with the V_H region of Igs [16], a property responsible for its SAg activity for B cells [1, 4]. To test the effect of SpA in vivo, we used V_H3+ Ig-specific SpA and 'XMG2L3' transgenic mice [17]. The mouse Ig loci of these rodents were inactivated and their humoral immune system has been restored by introduction of human V_H, V_κ and V_λ loci in a germline configuration. Their B lymphocytes express fully human surface Igs, and secrete IgM and IgG2 bearing κ or λ human L chains. A remarkable feature of these mice is that half of the V_H genes present on the translocus belong to the V_H3 gene family, a figure that closely mimics the complexity of the human Ig V_H locus. When immunized, they mount antigen-specific antibody responses with somatic mutation, isotype switching and affinity maturation [18]. As in the human serum [8], the V_H3+ IgG concentrations in the plasma of the transgenic

humanized mice were lower than V_H3+ IgMs. They represent approximately 40% of total IgG, probably reflecting the presence of somatic mutations in their V_H genes. We found that SpA treatment induces a rapid and specific decrease in plasma V_H3+ Igs, without affecting the total Ig concentrations [19].

B cell precursors expressing functional cell surface H chains in the bone morrow can be considered as potential targets for SpA, which is able to bind Ig H chains encoded by the V_H3 gene family. However, we demonstrated that repeated administration of SpA does not affect pro-B (B220lowCD43+IgM−), pre-B (B220lowCD43−IgM−), immature B cells (B220+IgM+IgD−), and mature recirculating cells (B220+IgM+IgD+) in the bone marrow. In future experiments, it will be important to test whether other B cell SAgs are able to impact B cell precursors in the bone morrow and cripple the humoral arm of the host.

Analyzing SpA effects in peripheral lymphoid organs, we found that a single injection of SpA to the transgenic humanized mice reduces B-1a cells in the peritoneal cavity (PeC) and V_H3+ B cells in the spleen. Importantly, even repeated administrations of this SAg do not modify B-1b or B-2 cell numbers [19]. The molecular mechanisms of the specific deletion of B-1a cells, as opposed to B-1b and B-2 cells, remain unresolved. They include alterations of homing, decreased production and/or developmental blockade of the corresponding progenitors, and accelerated cell death. In fact, we found that after only 16 h, SpA treatment triggers an important increase in apoptotic B cells in the spleen. It is also possible that the microenvironment of the peritoneum, and perhaps of other pleural cavities, provides a unique milieu that favors the induction of the hyporesponsiveness of B-1 cells. On the other hand, B cell receptor (BCR) ligation in the PeC may have different impacts on lymphocyte survival of B-1a and B-1b cells. Likewise, the lack of SpA effect on B-2 cells in the PeC may reflect their intrinsic resistance to BCR-mediated ligation in this microenvironment. Thus, the mechanisms that account for the observed diminution of B cells in treated mice require further investigation.

It is important to emphasize that B-1 cells play a unique role in the immune system. They produce the majority of natural antibodies (NAbs) and contribute substantial amounts of IgA [20]. Studies of mice lacking NAbs have demonstrated their essential role in providing early protection against a variety of pathogens [21–24]. In sum, NAbs represent an early B cell subset essential for 'housekeeping' responsibilities in the organism, targeting senescent red blood cells, oxidized membrane lipids, the products of apoptosis and intestinal microflora, and they play a role in innate immunity [25].

In the spleen of treated mice, we studied in detail the fate of various B cell subpopulations [26–29], including newly formed B cells (B220+CD23−CD21−), transitional T1 (B220+CD21+IgM+), transitional T2 (B220+CD21−IgM+),

recirculating follicular (FO; B220+CD23+CD21+) and resident marginal zone (MZ) B cells (B220+CD23−/lowCD21+). It is notable that MZ cells are located at the junction of the white and red pulps of the spleen, intimately associated with the marginal sinus. They secrete preferentially IgM and IgG, a reflection of their role in primary T-cell-independent responses. By contrast, FO B cells produce mainly IgG1 or other Ig isotypes, and engage in T-cell-dependent responses. Remarkably, we found that SpA induced a specific reduction of the MZ subpopulation in the spleen [19]. The molecular basis of this specific deletion of MZ B cells, as opposed to FO cells, remains unresolved. It is likely that the disparity in responses of MZ and FO B cells to SpA reflects a functional dichotomy in these two splenic subpopulations. In fact, it has become clear that MZ B cells represent a peculiar B cell subset that differs in many respects from the predominating subset of mature recirculating FO B cells [28, 30–40].

In single-cell PCR-based studies and sequencing of the expressed genes, we demonstrated that the reduction of MZ B cells in SpA-treated mice reflects the decrease in the absolute number of V_H3-expressing B cells in this subset [19]. Thus, the effect is a direct consequence of the V_H3+ B cell targeting capacity of SpA. To determine whether the SpA B cell deleting potential had functional consequences, we immunized SpA-treated mice with either a type 2 T-cell-independent antigen (DNP-Ficoll) or a T-cell-dependent antigen (tetanus toxoid). These studies disclosed a specific impact of SpA on the type 2 T-cell-independent response [19].

P. magnus Protein L Impairs Innate-Like B Cell Immunity

It is remarkable that, using another appropriate model of mice expressing human Igs, we found that protein L also targets B cells (MZ and B-1a) with innate-like functions [41]. In contrast to protein A, protein L from *P. magnus* binds predominantly to κ L chains, regardless of the H chain subclass, and it has affinity for all classes of Igs. Since approximately two thirds of human Igs have κ-type L chains, protein L interacts with a significant proportion of Igs. Its binding does not interfere with the antigen binding site and the affinity of the interaction ranges from $1.5 \times 10^9\ M^{-1}$ to $1 \times 10^{10}\ M^{-1}$, depending on the L chain and the H chain isotype [42]. Crystal structure of a human antibody Fab complexed through its V_L region with a protein L domain revealed that protein L interacts with the framework part of the variable regions without contacting the hypervariable loops [43]. In vitro, protein L acts as an SAg for human Igs. It cross-links the V_L domains of IgE bound to Fcε receptors, and stimulates the release of histamine by basophils and mast cells [44]. By acting as an SAg for human Igs, protein

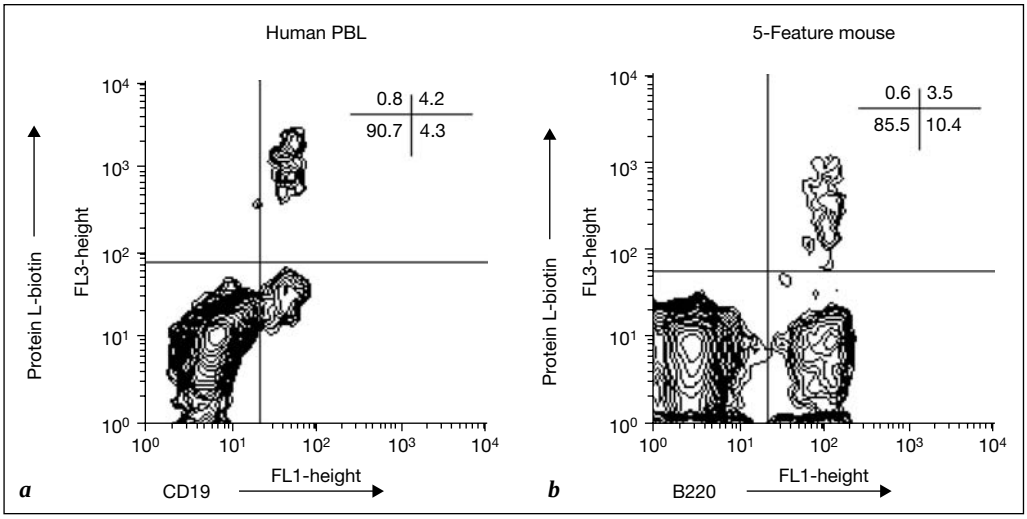

Fig. 1. Binding of protein L to human B cells and to mouse B cells expressing human Igs. ***a*** Human peripheral blood lymphoid cells were stained with FITC-labeled anti-human CD19 monoclonal antibody and biotinylated protein L, followed by Cy-chrome-labeled streptavidin. ***b*** Splenic cells from transgenic mice expressing human Igs [46] were stained with FITC-labeled anti-mouse B220 monoclonal antibody and biotinylated protein L, followed by Cy-chrome-labeled streptavidin. All monoclonal antibodies and their corresponding isotype controls were purchased from BD PharMingen.

L stimulates in vitro the release of preformed and de novo synthesized vasoactive and proinflammatory mediators (histamine, tryptase and cysteinyl leukotriene) by human mast cells isolated from heart tissue [45]. As shown in figure 1, protein L binds ex vivo to B cells expressing human κ L-chain-positive Igs.

To study protein L effects in vivo, we used '5-feature' transgenic mice [46]. In these rodents, the endogenous loci coding for the H and L chains have been inactivated, and human H, κ- and λ-chain transloci were 'knocked in'. The H chain translocus contains the core region of the human H chain locus with the five most 3′-located V_H gene segments, the complete D_H and J_H loci, and the Cμ and Cδ exons in correct germline configurations. The κ-chain translocus is a construct that contains 82 functional V_κ gene segments organized in 20 repeats of five V_κ gene segments linked to the core of the germline locus, including the first three V_κ gene segments, the complete J_κ cluster and the C_κ coding region. Finally, the λ-chain translocus encompasses 28 V_λ gene segments, including 16 functional genes, attached to the J_λ and C_λ coding regions in the correct germline configuration. Transgenic humanized 5-feature mice were injected intraperitoneally with 1 mg of recombinant protein L or the control

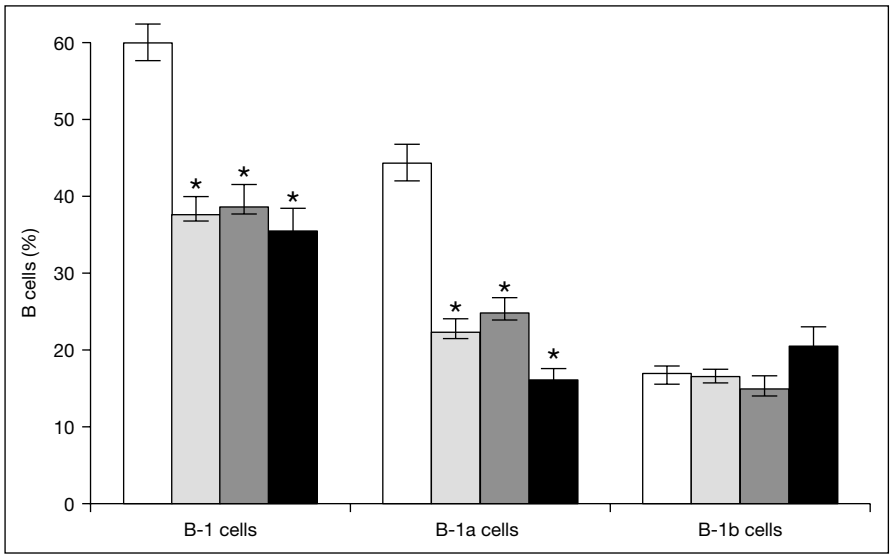

Fig. 2. Repeated injections of protein A deplete B-1a cells in the PeC. Transgenic Xenomouse mice expressing human Igs [17] were injected intraperitoneally with 0 (open bars), 125 (gray bars), 250 (stippled bars) or 500 μg (black bars) of recombinant protein A. After 24 h, peritoneal cells were washed and then cells were triple stained with fluorescent antibodies to human IgM (G20–127), mouse CD11b (M1/70), and mouse CD5-PE (53-7.3). FACS analysis allowed identification of the B-1a (IgM+CD11b+CD5+) and B-1b cell subsets (IgM+CD11b+CD5−). All monoclonal antibodies and their corresponding isotype controls were purchased from BD PharMingen. *$p < 0.05$.

protein hen egg lysozyme (HEL) on days 1, 3, 5, 7 and 9. On day 21, spleen cells were collected and their proliferative capacity was tested ex vivo. These studies disclosed that protein L injections did not alter the proliferation capacity of splenocytes (fig. 2). Investigating protein A effects on B cell subsets in the bone marrow, we found that injection of soluble protein A to these transgenic humanized mice results in a modest expansion of immature B cells (fig. 3). However, protein L-injected mice exhibited a loss of mature B cells in the peripheral immune system and a reduction of protein L+ B cells in the spleen (fig. 4). Despite the substantial reduction of B cells in the periphery, there is only a subtle, but not significant, decrease in the numbers of CD4+ and CD8+ T cells in the spleen and mesenteric lymph nodes. Further characterization of B cell subsets in protein-L-injected mice revealed a depression of MZ B cells in the spleen and a reduction of CD5+ B cells in the PeC [41]. By contrast, protein L treatment did not affect CD5+ B cell numbers in the spleen or the lymph nodes (figs. 5–7).

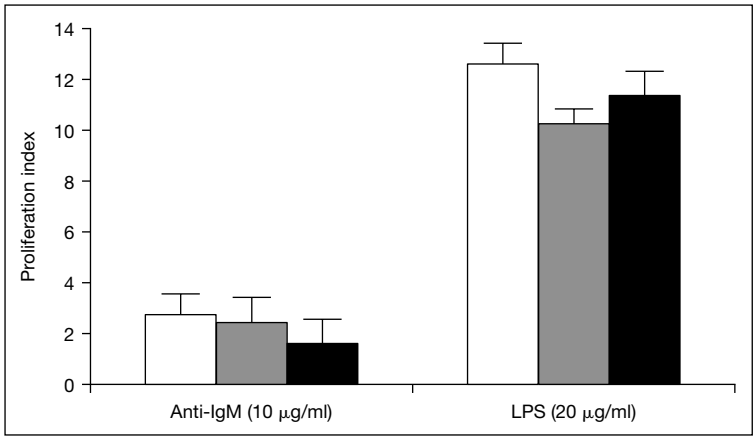

Fig. 3. Effect of protein L injection on the proliferation capacity of splenocytes. Transgenic 5-feature mice [46] were injected intraperitoneally with 1 mg of recombinant protein L or the control protein HEL on days 1, 3, 5, 7 and 9. Spleen cells were washed and then cultured at a concentration of 2×10^5 cells in 100 μl in 96-well, round-bottom plates for 18 h in the presence of $F(ab')_2$ anti-human IgM or lipopolysaccharide (LPS). Incorporation of ^3H-thymidine was measured and proliferation indexes were calculated. White bars = Control, non-injected mice; gray bars = mice injected with HEL; black bars = mice injected with protein L.

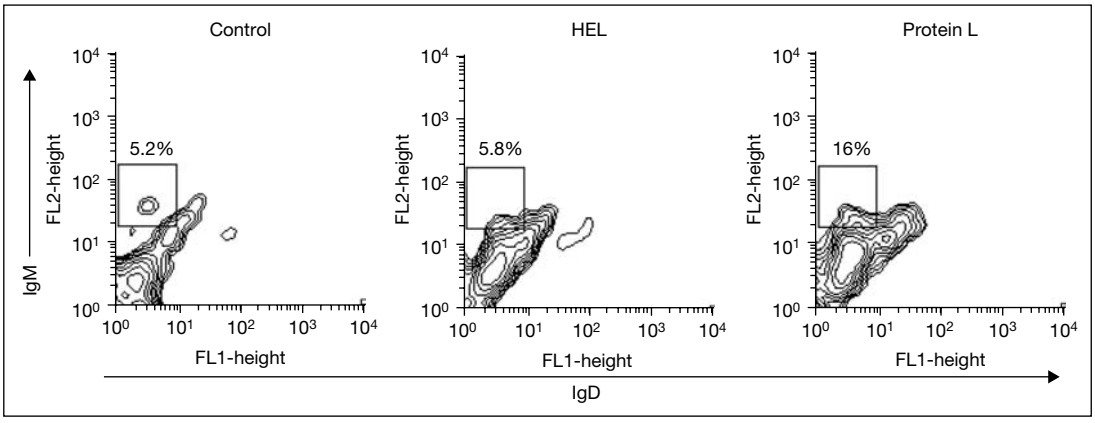

Fig. 4. Immature bone marrow lymphocyte (B220+IgM+IgD−) numbers are increased in protein-L-treated transgenic mice. Transgenic mice expressing human Igs [46] were injected intraperitoneally five times with 1 mg of either protein L or the control protein HEL every other day. On day 21, femurs and tibias were ground in a mortar and filtered through sterile cotton to remove bone fragments. Cells were triple stained with Cy-chrome-labeled anti-mouse B220 monoclonal antibody, phycoerythrin-labeled anti-human IgM monoclonal antibody and FITC-labeled anti-human IgD monoclonal antibody. Shown are lymphoid cells gated on the B220-expressing cells in control, HEL- and protein-L-treated mice. All monoclonal antibodies and their corresponding isotype controls were purchased from BD PharMingen.

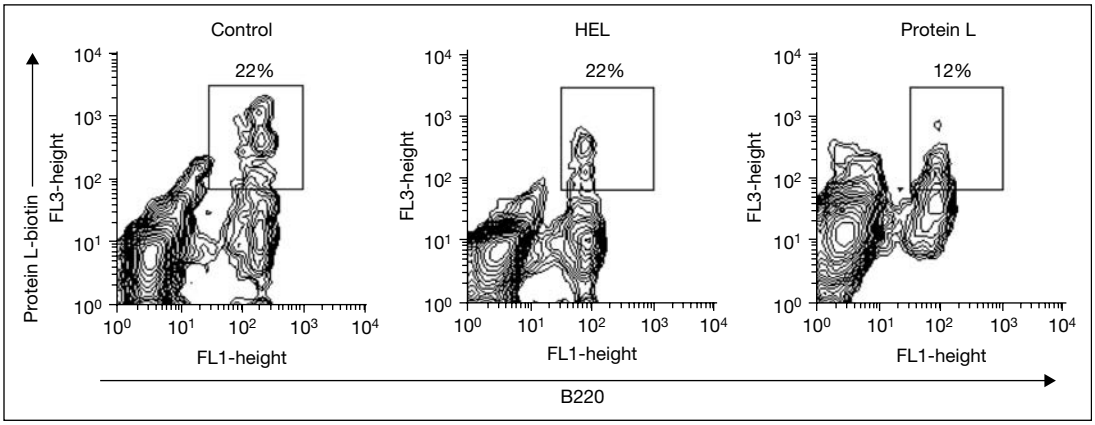

Fig. 5. Specific marked reduction of the number of protein-L-reactive cells in the spleen of transgenic humanized mice. Transgenic mice expressing human Igs [46] were injected intraperitoneally five times with 1 mg of protein L every other day. On day 21, splenic cells of control, HEL- and protein-L-treated transgenic mice were depleted of erythrocytes using Tris-buffered ammonium chloride and stained with cell surface markers. B220+ protein L+ cells are detected by FITC-labeled anti-mouse B220 monoclonal antibody and biotinylated protein L, followed by Cy-chrome-labeled streptavidin. All monoclonal antibodies and their corresponding isotype controls were purchased from BD PharMingen.

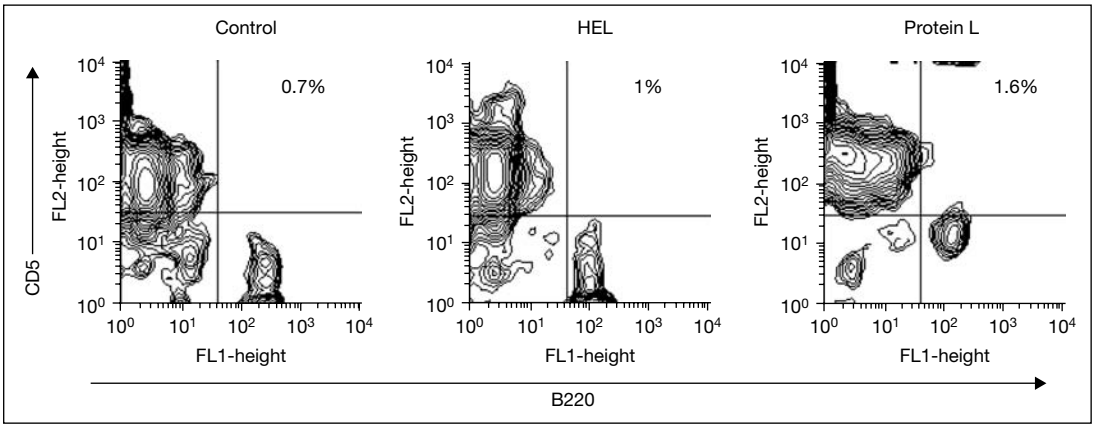

Fig. 6. Effect of protein L injections on CD5+ B cells in the spleen. Transgenic mice expressing human Igs [46] were injected intraperitoneally five times with 1 mg of protein L every other day. On day 21, splenic lymphoid cell subpopulations were identified by immunofluorescent staining in control, HEL- and protein-L-treated transgenic mice. Splenocytes were stained with Cy-chrome-labeled anti-mouse B220 monoclonal antibody and phycoerythrin-labeled anti-mouse CD5 monoclonal antibody. All monoclonal antibodies and their corresponding isotype controls were purchased from BD PharMingen. The treatment did not affect B1 cells in the spleen.

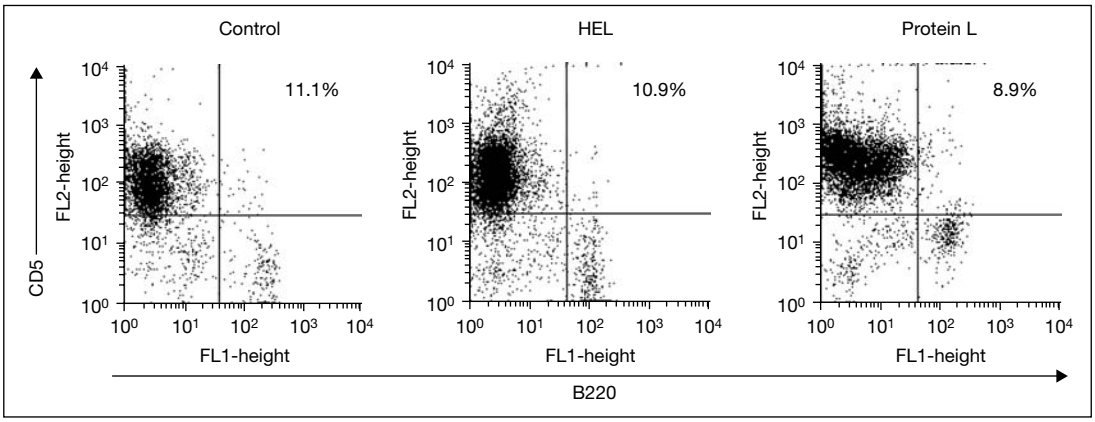

Fig. 7. Effect of protein L injections on CD5+ B cells in lymph nodes. Transgenic 5-feature mice expressing human Igs [46] were injected intraperitoneally five times with 1 mg of protein L or the control protein HEL on days 1, 3, 5, 7, and 9. On day 21, mesenteric lymph node cell subpopulations were identified by immunofluorescent staining in control, HEL- and protein-L-treated transgenic mice. Cells were stained with Cy-chrome-labeled anti-mouse B220 monoclonal antibody and phycoerythrin-labeled anti-mouse CD5 monoclonal antibody. All monoclonal antibodies and their corresponding isotype controls were purchased from BD PharMingen. The treatment did not affect B1 cells in lymph nodes.

Depleting Activity of B and T Cell Superantigens

Accumulating evidence indicates that activation-induced cell death of T cells provides a mechanism for the deletion of antigen-activated T cells, thereby leading to the resolution of inflammation and playing an important role in maintaining homeostasis of immune responses [47]. It is therefore of interest to compare the in vivo effects of B cell and T cell SAgs. These latter ligands differ from conventional peptide antigens in that they bind to major histocompatibility class II molecules outside the peptide groove, and that they are not processed into small peptides by antigen-presenting cells. Specifically, they interact with the T cell receptor (TCR) via amino acid residues encoded by the Vβ genes of the TCR rather than with the highly variable, non-germline-encoded residues of the TCR β- and α-chain third hypervariable regions. As a result, large percentages of T cells expressing the same Vβ genes are targeted. In vivo, they cause polyclonal T cell activation and cytokine release, often followed by T cell apoptosis or anergy [5]. T cell SAgs can either transiently activate T cell subsets or lead to their functional demise. The targeted T cell subsets may contain helper T cells (Ths), regulatory T cells or autoreactive aggressive

T cells. It is therefore difficult to predict the clinical outcome of SAg encounter. For instance, staphylococcal enterotoxin B (SEB) stimulation in vitro results in sequential T cell activation, proliferation, CD95 expression, cytokine secretion, and is subsequently followed by apoptosis in a dose- and time-dependent manner [48]. It induces upregulation of CXCR3+ cells in SEB-reactive CD4+ T cells from healthy subjects and of CCR4+ cells in those from atopic dermatitis patients [49]. It also induces apoptosis of both CXCR3+CD4+ and CCR4+CD4+ T cells from healthy individuals. The CXCR3+CD4+ T cells are reportedly more susceptible to SEB-induced apoptosis than CCR4+CD4+ T cells in healthy subjects, although they have a similar susceptibility to spontaneous apoptosis [49].

While the observations summarized above might imply that Th2 cells are more resistant to SEB-induced apoptosis than Th1 cells in subjects with normal cytokine balances, studies of polarized T cell lines indicate that Th2 cells are more resistant to activation-induced cell death than Th1 cells [50, 51]. Consistently, T cell responses to SAgs usually involve Th1 cells and release of the cytokines IL-2, TNF-β, and INF-γ, rather than the usual pattern of induction of a Th2 response by exotoxins, which leads to the production of antibody to the exotoxin. It remains to be seen whether B cell SAgs target preferentially Be1 or Be2 cells [52].

Exploitation of Innate B Cell Functions by B Cell Superantigens

The observations summarized above indicate that microbial SAgs preferentially impact B cell subpopulations responsible for innate-like functions. In contrast to FO B cells, also called B2 cells, the B-1a and MZ B cell subsets have evolved to provide a first line of defense for antigens acquired through the gut/peritoneum and the blood stream [28, 53]. Like B-1 cells, MZ B cells are poised to provide a first line of response to pathogens and form an integrated part of innate immunity [54, 55]. Because of their location, MZ B cells are able to efficiently encounter and respond to blood-borne pathogens. On the one hand, mature recirculating FO B cells have evolved to generate a huge repertoire able to mount T-cell-dependent B cell responses with high affinity and long-term memory [56]. On the other hand, MZ B cells have a slower turnover rate compared to FO B cells and do not appear to recirculate. They differ from FO B cells in phenotype, differentiation potential and responsiveness to T-cell-independent antigens. Thus, MZ B cells express high levels of CD21 and CD38, molecules that have been shown to lower the threshold of BCR responses, making MZ B cells more reactive. Analysis of mice lacking MZ B cells has led to the suggestion that recruitment into this population is regulated by the strength

of BCR signaling and that it can be modulated by coreceptors such as CD19 [54, 57]. MZ B cells can become quickly activated in response to stimuli and can proliferate, secrete Ig and flux Ca^{2+} more rapidly and to a higher magnitude than FO and newly formed splenic B cells [35]. However, proliferation induced by cross-linking of CD38 or IgM is greater in FO B cells than in MZ B cells. Given the compelling similarities between B-1 and MZ B cells [58], our data suggest that a common set of factors are important for the deletion of both of these populations by B cell SAgs [19, 41].

Conclusions

It is clear that the study of SAgs has substantially increased in prominence recently. As can be gleaned from the discussion above, there is a very rapidly expanding literature describing a broad array of previously unknown SAg activities that are capable of impacting the immune system. For the SAgs described here, there is reasonable indication, based on specificity and on the effects observed in vivo in experimental animals, that certain pathogens have evolved primarily to subvert immune functions. Nonetheless, the evolution of pathogens and their hosts are inextricably intertwined, and studies of SAgs have uncovered new and interesting dimensions in the complex and ongoing battle between pathogens and their hosts. It is important to emphasize that we are constantly exposed to certain SAgs. For example, *S. aureus* commonly colonizes the nose of normal subjects. This triggers an antibody response to the protein A SAg, and these antibodies may play a protective role against the SAg effects of SpA. Conversely, SAgs could anergize critical B cell subsets that would otherwise protect the host from disease. As discussed above and elsewhere [19, 41], B cell SAgs can deplete B cell subsets in vivo. Beyond the scope of host-pathogen interactions, this B cell SAg depleting potential could be useful for immunointervention purposes, including autoimmune diseases. Thus, we found that weekly intraperitoneal injections of SpA delay the progression of serum anti-DNA IgG and reduce proteinuria early in young female (NZBxNZW) F_1 mice, an experimental model of systemic lupus [59]. In sum, the potential significance of SAgs is considerable. Perhaps we are just beginning to understand how pathogens can attack B cells [60].

Acknowledgements

This work was supported by grants from the Agence Nationale de Recherches sur le Sida (ANRS, Paris). Thanks to Muriel Viau for expert contributions, to Dr. Lars Björck (Lund University, Sweden) for providing recombinant protein L, to Drs. Marianne

Brüggemann (The Babraham Institute, Cambridge, UK) and Larry Green (Abgenix, Inc., Fremont, Calif., USA) for providing transgenic mice expressing human Igs.

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Marone G (ed): Superantigens and Superallergens.
Chem Immunol Allergy. Basel, Karger, 2007, vol 93, pp 106–136

The Allergic March from *Staphylococcus aureus* Superantigens to Immunoglobulin E

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Abstract

Staphylococcus aureus is a commensal bacterium in the respiratory tract mucosa of most people and infects the skin of atopic dermatitis patients. This might imply a symbiotic relationship between host and bacterium or a standoff between bacterial infection and the host immune system. But superantigens produced by *S. aureus* in these locations are of particular interest because they are strongly implicated in the pathogenesis of allergic disorders and airway disease. They appear to act locally in these conditions by stimulating polyclonal T cell and B cell proliferation and driving somatic hypermutation, class switching to immunoglobulin (Ig) E and the production of allergen-specific IgE in mucosal B cells. IgE antibodies directed against the superantigens ('superallergens') themselves engender chronic inflammation and the persistent sensitization to conventional allergens of mast cells and antigen-presenting cells in mucosal tissues in atopic dermatitis, rhinitis and asthma. Moreover, *S. aureus* superantigens inhibit the activity of T regulatory cells that normally control inflammation, and generate a state of steroid resistance that confounds treatment of allergic disorders and airway disease.

Immunoglobulin (Ig) E makes up one of the nine antibody classes in man and is a central player in allergic hypersensitivity and allergic inflammation (reviewed in Gould et al. [1]). The concentration of IgE in serum (<1 μg/ml) is less than one ten thousandth that of IgG (10 mg/ml), and thus it is the least abundant antibody class in the circulation. But this does not necessarily imply that comparably small amounts are produced in the body, for IgE is both

synthesized and sequestered in mucosal tissues, where it also exerts its effector functions [2]. It is sequestered by the mucosal mast cells by virtue of its extremely high affinity for the receptor, FcεRI. The affinity of IgE for FcεRI ($K_a = 10^{10}-10^{11}$ M^{-1}) is 2–5 orders of magnitude greater than that of IgGs for their receptors ($K_a = 10^6-10^8$ M^{-1}). The half-life of IgE bound to mast cells in tissues is about 2 weeks. Mast cell precursors migrate to mucosal tissues and express FcεRI there at a high level (10^5-10^6 molecules/cell). FcεRI is also expressed at lower levels on Langerhans cells and dendritic cells, which exercise a surveillance function by taking up antigens in the epithelial and mucosal tissues, and thereafter migrate to local lymph nodes and present them to T cells. It is thought, therefore, that most of the IgE in the body is sequestered by the mast cells in mucosal tissues. Some of the IgE-expressing B cells may escape into the circulation and populate other tissues, and the huge excess of IgE produced by B cells in mucosal tissues (over that required to saturate IgE receptors on the IgE effector cells) may be the source of some of the circulating IgE [1].

An allergic response occurs when a multivalent antigen, becoming an allergen, cross-links FcεRI-bound IgE antibodies on mast cells. The allergen-activated mast cells exhibit immediate hypersensitivity, releasing various mediators of physiological changes in the local vasculature and musculature, together with a mixture of cytokines and chemokines. These in turn recruit inflammatory cells from the circulation and activate the cells to participate in a later response and, if allergen stimulation persists, in chronic inflammation.

The designation ‘superantigen’ refers to the power of these antigens not only to mimic, but also to exceed the activity of conventional antigens [3–5]. Superantigens induce polyclonal proliferation of subsets of T cells and B cells, by interaction with conserved features of their antigen receptor structures, rather than their conventional antigen binding sites, and thereby induce polyclonal proliferation or deletion; this effect is associated with a wide range of disease syndromes from autoimmune and allergic conditions to neoplasia and immunodeficiencies [6, 7]. Excessive proliferation can cause anergy or apoptosis, depending on such factors as the superantigen dosage and the cell lineage, stage of development and microenvironment. Clearly in autoimmune and allergic diseases, cell proliferation may result in breaking tolerance, with the result that autoantibodies (of the IgM and IgG classes) and anti-allergen (IgE) antibodies might be produced [8].

Association of *S. aureus* Superantigens with Allergic Disease

S. aureus is not the only potential source of superantigens in mucosal tissues, the primary targets of allergic disease. We focus here on this bacterium because of

its prevalence in mucosal tissues, the many well-characterized superantigens (*S. aureus* enterotoxins or SEs) that it produces [9], and the wealth of evidence implicating these superantigens in the pathophysiology of allergic diseases.

The link between SEs and allergic disorders is most firmly established for atopic dermatitis (AD) (reviewed in Leung [10] and Breuer et al. [11]). AD affects 10–15% of the population, but *S. aureus* colonization is observed in the skin lesions of >90% of AD patients, compared with 5% of normal skin [12]. Moreover, the intensity of skin inflammation has been correlated with the degree of *S. aureus* colonization [13]. Most strains of *S. aureus* grown from atopic skin have been shown to produce SEs with superantigenic properties [14]. In accordance with the view that the SEs induce or exacerbate AD, treatment of AD patients with steroids in combination with antibiotics is more effective than treatment with steroids alone [15, 16].

The association of SEs with airway disease is also robust (reviewed in Bachert et al. [17]). Some 20% of the human population are permanently, and 60% intermittently colonized by *S. aureus* in their nasal mucosa [18]. Asthma affects around 20% of the population at some stage of life [19]. *S. aureus* colonization of the nasal cavity is significantly higher in house-dust-mite-allergic patients with perennial allergic rhinitis compared to normal controls [20] and this has been associated with increased nasal IgE levels [21]. IgEs specific to *S. aureus* enterotoxin A (SEA), SEB, SEC, SED and toxic shock syndrome toxin-1 (TSST-1) have been reported in the serum of both allergic rhinitis and asthma patients at higher incidence than in normal controls and specific IgE levels were correlated with disease severity [22–24]. This is surmised to be why antibiotics have proved to be a useful adjunct to steroids and β-agonists in the treatment of asthma [25].

Targets of *S. aureus* Enterotoxin Activity

Superantigen target three key proteins of the adaptive immune response, namely the T cell receptor (TCR), the B cell receptor (BCR) and major histocompatibility complex class II (MHC II) proteins. All are members of the Ig superfamily, and contain domains of similar tertiary structures, although MHC II contains two non-Ig-like domains. The structure of the Ig-like domain consists of two β-sheets, each of which comprises a series of anti-parallel β-strands, connected by loops of varying length. Ig domain structures embody two separate functions; the first is the association with antigens, with each other and with other proteins, such as Fc receptors or complement, while the second is maintenance of the structural integrity of the domain itself.

There are two types of superantigens: T cell and B cell superantigens.

T Cell Superantigens

T cell superantigens simulate the activity of conventional antigens by mediating the interaction between T cells and antigen-presenting cells (APCs), including monocytes, dendritic cells and B cells (reviewed in Kotzin et al. [5]). Conventional antigen presentation involves recognition by the TCR at the surface of T helper (Th) cells, of a specific antigenic peptide, presented in the context of an MHC II protein on the APC membrane. The subsequent interaction of the activated Th cells with cognate B cells, recognizing native antigens via the BCR, leads to proliferation of the B cells, which go on to produce antibodies directed against the antigen in the immune response.

The TCR is an integral membrane protein comprising an α- and a β-chain, each possessing two extracellular Ig-like domains. The membrane-distal, N-terminal 'variable' (V) domains of the two chains participate in binding to the MHC-II-peptide complex on the APC, recognizing both moieties. The TCR V domains are expressed after somatic recombination of one each of two (α-chain) or three (β-chain) germline gene segments, Vα and Jα, and Vβ, Dβ and Jβ, respectively, in thymic T cell precursors. Each of the recombined segments belongs to a large family of similar germline segments comprising a V, D or J family. In addition to the combinatorial diversity generated in this fashion, nucleotides can be added at the junctions during DNA recombination. The resulting junctional diversity makes by far the largest contribution to the total diversity in the T cell repertoire. It is estimated that the human T cell repertoire contains 10^{16} unique specificities, and considerably fewer than one in a thousand T cells are generally able to recognize a particular antigenic peptide [26].

Some of the highly conserved sequences of the TCR V domains, those that maintain the structural integrity of the Ig domains, include residues that face outwards into the solvent. Superantigen binding sites have been located in such regions of the TCR β-chains, encoded by 1 of the 52 Vβ gene segments [26]. The relatively small number of Vβ gene segments, compared to the size of the repertoire of antigen binding specificities, accounts for the ability of T cell superantigens to stimulate a massive proliferation of non-antigen-specific T cells, especially since many of the superantigens recognize more than 1 of the 52 Vβ sequences. Polyclonal stimulation of T cells in turn stimulates the polyclonal proliferation of B cells.

MHC II proteins are likewise integral membrane proteins comprising an α- and a β-chain, each with two extracellular domains. The peptide is held in a groove between the membrane-distal, N-terminal (non-Ig-like) domains of the two chains. An individual can generate eight different MHC II peptide binding specificities, since there are four types of matched α- and β-chain gene pairs in the HLA-DR, HLA-DP and HLA-DQ loci, and contributions from the maternal

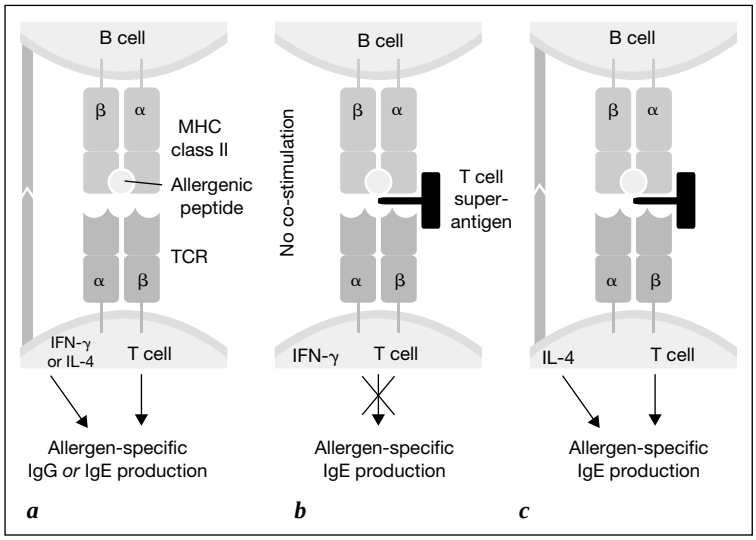

Fig. 1. How a T cell superantigen stimulates an antigen-specific response (adapted from fig. 5 in Hofer et al. [59]. ***a*** Presentation of a conventional antigen with costimulation (thin vertical bars connecting the B cell or APC and T cell). ***b*** Superantigen enhancement of allergen presentation in the absence of costimulation. ***c*** Presentation of the same allergen as in ***b*** with costimulation.

and paternal chromosome [26]. Additional specificities are present within the population, owing to the high degree of polymorphism at these loci; there are about 20 polymorphic positions in each chain, and some loci can accommodate up to 72 alleles [26]. Recognition of the MHC II proteins by superantigens tends to be unrestricted because the polymorphic residues generally lie in the peptide-binding groove, where they govern the peptide binding specificity, rather than on the surface of the protein, where the superantigen binding site is located.

In the interaction between cognate T cells and APCs, the TCR and MHC II proteins are always aligned in the same manner, with the TCR β-chain facing the MHC II molecule α-chain and vice versa. T cell superantigens attach first to their binding site on the MHC II α-domain, and then to the TCR β-domain. The order of binding could be due to the higher affinity of the superantigen for the MHC II protein than for the TCR. The TSST-1/HLA-DR interaction has a K_d of 10 nM [27]. Figure 1a and b compares schematically the 'cognate' interactions between T cell and APC, as mediated by superantigens and by conventional antigens. This model, originally proposed by Marrack and Kappler [4] and Kotzin et al. [5], is well supported by protein crystallographic studies of superantigen structures and the actual or modelled structures of their binary or ternary

complexes. The recent crystal structure of SEH in complex with MHC II proteins has revealed a zinc-dependent binding site in the MHC II β-domain [28]. It remains to be seen whether other SEs with zinc-dependent binding sites also bind to the MHC II β-chain. The crystal structures also reveal more subtle effects, such as partial occlusion of the peptide binding site in some cases [29, 30] and interaction with the backbone of the MHC-II-bound peptide in others [31].

S. aureus is a source of multiple T cell superantigens, which include SEA, SEB, SEC_1, SEC_2, SEC_3, SED, and SEE to SEQ, and TSST-1 [9]. SEA to SEE and TSST-1 are probably the best-characterized SEs in respect of their link to IgE and allergic disease. Common strains of this bacterium produce several of the enterotoxins simultaneously. Furthermore, each of them may recognize more than one Vβ sequence. SEB, for example, can bind to Vβ1.1, Vβ3.2, Vβ6.4 and Vβ15.1, whereas TSST-1 binds only to Vβ2.1 [9]. The Vβ chains and MHC II molecules recognized by SEA to SEE, SEI, SEK, SEQ and TSST-1 and others are also known [4, 9]. TSST-1 can stimulate up to 50% of all αβ+ T cells [4], causing food poisoning and the frequently fatal toxic shock syndrome.

At least two of the *S. aureus* T cell superantigens, SEA and SED, are also characterized as B cell superantigens, which we discuss in the next section.

B Cell Superantigens

Cognate interaction between Th cells and B cells is required to activate naïve B cells in the primary immune response; but after immunological memory has been established, cross-linking of the antigen receptor in the cell membrane may suffice to stimulate cell proliferation. B cell superantigens bind to the solvent-accessible, conserved regions of the V domain regions of the BCRs, analogous to the mode of binding of T cell superantigens to TCR V domains.

Antibodies, whether the membrane-bound BCRs or secreted forms, comprise two identical light (L) and heavy (H) chain pairs, with two Ig domains in the L chain and four or five (depending on the isotype) in the H chain. The two antigen binding sites are located in the membrane-distal, N-terminal V domains between L and H chain pairs. As for the TCR α- and β-chains, two gene segments, V_L and J_L, encode the L chain V domain (V_L) and three gene segments, V_H, D_H and J_H, the H chain V domain (V_H). The expressed V domain genes comprise one from each of the V, D and J germline gene families to generate combinatorial diversity, while nucleotide and palindrome addition generates additional junctional diversity in a manner analogous to recombination in the TCR locus. Such is the specificity of the junctional sequences that they can uniquely identify B cell clonal families derived from a single progenitor cell; this is important in discriminating between clonal families that encompass

clones differing elsewhere in their V region sequences in consequence of somatic hypermutation (SHM) [32].

SHM generates additional diversity in the BCR by introducing point mutations into the rearranged V domain sequences. SHM generally occurs in three regions termed 'complementarity-determining regions' (CDRs), which encode discontinuous protein sequences that form the conventional antigen binding sites in the three-dimensional structure of the protein. The targeting of the CDR-coding sequences is determined, at least in part, by the distribution of certain sequences, (A/G)G(C/T), preferred by the enzyme activation-induced cytidine deaminase (AID), involved in the process of SHM. This B-cell-specific enzyme deaminates cytidine residues in DNA, and the resulting uracils are targets for DNA strand breakage, involved in the process of SHM [33], as well as for class switch recombination (CSR) [34]. The three CDRs are flanked on all sides by four 'framework regions' (FRs) in both the L and H chains of the V domains. The more highly conserved FRs maintain the structural integrity of the Ig V domains.

Th-cell-activated naïve B cells compete for intact antigen presented by follicular dendritic cells in the germinal centers of lymphoid tissue. The B cells expressing antibodies with the highest affinity for the antigen are rescued from apoptosis (the fate of the vast majority of the B cells that circulate through the lymphoid tissue) by a process called 'affinity maturation'. When all sources of diversity, including the random pairing of different combinations of H and L chains, are taken into account, the potential B cell repertoire runs to 10^{18} unique specificities [26].

In contrast to conventional antigens, which bind to both the H and L chain CDRs at the distal end of the V domains, B cell superantigens bind to the more conserved FRS on the sides of either the H or L chain [35–37]. This can result in the massive proliferation of B cells. The 51 functional V_H gene segments can be arranged into seven families of varying size, within each of which the sequences are around 80% homologous. The size of the families varies from 22 functional members for the V_H3 family to 1–2 members for V_H5, V_H6 and V_H7 families [38]. In the normal blood B cell repertoire, expression of the different V_H families corresponds roughly to their size [39]. Two staphylococcal B cell superantigens, *S. aureus* protein A (SpA) as well as SEA, mentioned above as a T cell superantigen, bind specifically to V_H3, whereas another SE (SED), which is also a T cell superantigen, binds specifically to V_H4 [6]. Certain B cell superantigens target the C domains of Igs, but these will not concern us here.

SpA, the archetypal B cell superantigen [36], contains five homologous tandemly repeated domains, each of which can bind to all or most of the V_H3-containing BCR or Ig expressed by B cells. The nature of the SpA-V_H3 complex was established by high-resolution crystallography [40]. As V_H3 is the

largest of the seven human Ig germline V_H families, SpA can stimulate almost half of the B cells in the circulation. SpA can thus create a bias in the B cell repertoire, either towards increased V_H3 expression, due to selective expansion of the family, or its deletion. The latter may result in the loss of important protective antibodies of the V_H3 family, causing immunodeficiency [6].

SpA is expressed during the exponential growth of *S. aureus.* The commensal bacterium in mucosal tissues is maintained in a latent state, which could have beneficial effects (e.g. the formation of a pool of B cells that can act in the first-line defense against pathogens [41]) as well as harmful effects (due to B cell death) in vivo. Limiting dilution cloning of *S. aureus* from the skin of patients with chronic AD reveals that there can be 10^7 colony-forming units/cm^2 of skin [42], and that they can secrete a mixture of SEs [43]. The SEs fall into subgroups based on sequence homologies [9]. However, they all possess two dissimilar domains, rather than several similar domains as in SpA, and should thus, in principle, be unable to cross-link V domains on the surface of B cells.

SEA and SED are the two SEs known to exhibit B cell superantigen activity by binding to Igs expressing specific V_H families, V_H3 and V_H4, respectively [44, 45]. SEA binds more weakly than SpA to V_H3 (K_d = 10 μM, as against 60 nM), reflecting the faster rate of dissociation of its complex with V_H3 [30]. The greater strength of SpA binding is probably due to the affinity of its sites on five domains, which, upon binding to multiple V_H3 domains, may result in clustering of BCRs on the membrane of B cells. Unlike SpA, neither SEA nor SED stimulate the proliferation of isolated B cells in vitro, but they do enhance the survival of the V_H3- or V_H4-expressing B cells [30, 31]. This could be important in determining the B cell repertoire in tissues that can accommodate only a fixed number of proliferating B cells.

Silverman and Goodyear [36] have suggested that encounters with a B cell superantigen result in activation-induced cell death because of the enhanced consumption of cytokines, such as IL-4 and CD40 ligand (CD40L), required for survival and/or cell cycle progression. They point out that this exposure, as it affects a sizeable proportion of cognate B cells, would lead to increased cellular competition and selective survival of these superantigen-binding B cells (a deviant form of affinity maturation), manifested by the abundance of V region clonotypic BCR markers [6].

Mechanism of Antibody Class Switching to IgE

The nine antibody classes in man, IgM, IgD, IgG1–4, IgA1, IgA2 and IgE, differ in their Ig H chain constant (C_H) regions. These are encoded in a tandem

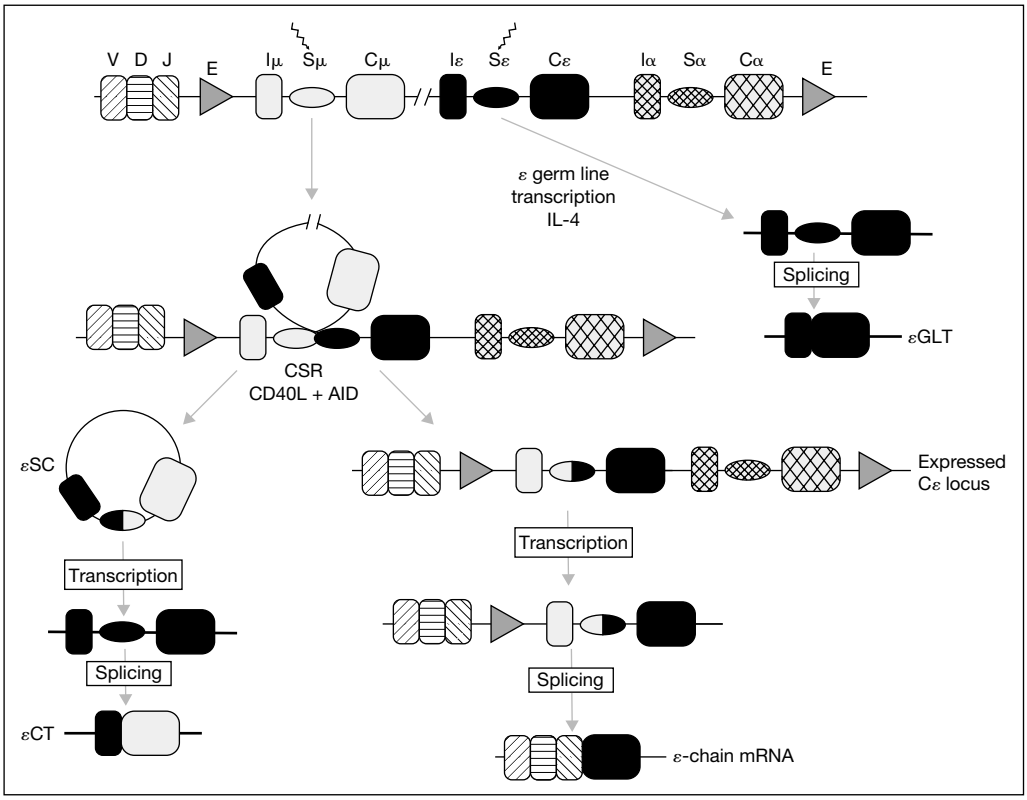

Fig. 2. Mechanism of CSR to IgE. Human Ig H chain C region germline genes are organized in a tandem array downstream from the expressed variable (V_H or VDJ) genes on chromosome 14. Each C_H class gene comprises an intervening exon, a switch region, containing the potential DNA breakpoints (zig zag arrows), and the C_H exons. The different elements are pattern coded to follow the process of CSR. IL-4 (or IL-13) is required for ε germline gene transcription, which is essential, along with CD40L and AID, for CSR to IgE. The recombined ε gene is transcribed into an ε-chain mRNA precursor, which is then spliced to form ε-chain mRNA. A by-product of CSR to IgE is an εSC, in this example Iε-Cμ, which is transcribed and spliced into the εCT. εSCs and εCTs are used as markers for direct CSR from IgM to IgE. Similarly, γSC and γCT and αSC and αCT are used as markers for sequential switching from IgM to IgG or IgA, respectively, and then to IgE.

array on human chromosome 14, downstream from the V_H germline gene families (fig. 2). IgE is characterized by its ε heavy chains (V_H-Cε), which combine with the L chains expressed in a B cell to form the finished antibody. All B cells first express IgM because the expressed V_H region is originally linked to Cμ, encoding the μ-chain of IgM (fig. 2). V_H must recombine with Cε to express

the ε-chain of IgE. CSR retains the antigen specificity of the antibody, but changes the antibody class and therefore the effector functions of the antibody classes. Class-specific Ig receptors are expressed in different cell types and different ratios in these cell types, located in varied anatomical compartments, thereby diversifying the effector functions of antibodies.

An intervening sequence exon and a switch region lie upstream from each C_H gene on chromosome 14 (fig. 2). Germline gene transcription of the recombining C_H genes is required for CSR. Transcription is initiated from the intervening exon promoter and proceeds through the switch and C_H regions. The germline gene transcripts (GLT) cannot be translated into protein because the intervening exons contain stop codons in all 3 reading frames, but they participate in the mechanism of CSR. Transcription is activated by specific T cell cytokines, secreted during cognate interaction between activated Th cells and B cells.

The circumstances of Th cell activation determine which of the two pathways of Th cell differentiation follows, leading into the Th1 or Th2 lineage [46]. Antigen-activated Th2 cells secrete the cytokines, IL-4 and/or IL-13, required for transcription of the ε germline gene and class switching to IgE (reviewed in Romagnani and Del Prete [47] and Gould et al. [48]). Th1 cells secrete IL-2 and IFN-γ, which promote class switching to IgGs, but not to IgE. The two cytokines, IL-4 and IFN-γ, are mutually antagonistic; each inhibits the generation of the other's Th cell. Their activities tend to polarize the T cells into one or the other lineage and influence which cytokines are delivered to B cells and therefore the direction of class switching in different anatomical compartments.

IL-4 is also required as a necessary proliferation stimulus to the B cell that enables the expression of IgE. Class switching is linked to cell division, and more cycles of cell division are needed for switching to IgE than to IgG [49]. In addition, IL-4 upregulates the production of AID, which catalyses both CSR and SHM. It catalyses the deamination of cytidines in the switch regions of the Ig H chain gene locus to facilitate DNA breakage and subsequent nonhomologous end joining [34, 50]. IL-4 and/or IL-13, εGLT and AID are therefore necessary (but not sufficient) for CSR to IgE in B cells.

CD40L is the additional signal required for CSR (reviewed in Gould et al. [48]). CD40L binds to CD40 on B cells. The interaction of IL-4 with the IL-4 receptor, and CD40L with CD40 on the B cell, induce the expression of costimulatory molecules required for CSR on both cell types, e.g. CD28/CTLA-4 (T cell) and B7.1/B7.2 (B cell) [51, 52]. DNA is cleaved in the two switch regions and a hybrid switch junction is formed to express the new γ-, α- or ε-chain mRNA (fig. 2). A by-product of this reaction is the looped-out and deleted intervening sequence, the ends of which join to form the reciprocal hybrid junction in a circular DNA. Each recombination event yields only one

switch circle (SC), which does not undergo replication and is lost upon clonal expansion of the B cells.

However, SCs are transiently transcribed from the deleted intervening exon promoter, now linked to the previously expressed C_H, thus providing a marker, a switch circle transcript (CT) for CSR.

Both the SC and CT have been used as markers for specific events in CSR, after cloning and sequencing the DNA or cDNA [53, 54]. Class switching to IgE may occur directly from IgM or sequentially via one or more of the IgG subclasses and/or IgA1. IgE-expressing B cells may in turn switch only to making IgA2, but not vice versa, because of the order of C_H genes in the H chain locus and deletion of the intervening sequence that accompanies each round of CSR (fig. 2).

Class Switching to IgE and Synthesis of Allergen-Specific IgE

SEs may stimulate or inhibit IgE synthesis by peripheral blood mononuclear cells (PBMCs) in vitro depending on conditions. The concentrations of the superantigens and cytokines (IFN-γ and IL-4), the source of the cells (whether from atopic or normal donors), and the ratio of T cells and B cells in reconstituted mixtures influence the outcome with respect to the synthesis of total IgG and IgE and allergen-specific IgE.

Mourad et al. [27] measured the affinity of TSST-1 binding to B cells (K_d = 30 nM) and the saturation level (76,000 sites/cell), and demonstrated that anti-HLA-DR and -DQ antibodies inhibit the interaction between TSST-1 and the MHC II proteins. They showed that TSST-1 at 1 μg/ml stimulates the proliferation of highly purified B cells after reconstitution with irradiated CD3+ T cells at an optimal T cell:B cell ratio of 1:1. TSST-1 also induced T-cell-dependent differentiation of B cells into IgG- and IgM-secreting cells at an optimal T cell:B cell ratio of 1:5. Using the appropriate blocking antibodies, they demonstrated that cell contact between MHC II proteins and the TCR-CD3 complex are critical for the effects of TSST-1 to emerge. They observed that several other cell surface proteins (e.g. CD4 on T cells) that participate in the presentation of conventional antigens were unnecessary for TSST-1 activity. Furthermore, irradiated cells from a T cell line acted as efficiently as syngeneic primary CD4+ T cells, proving that T cell superantigen binding to T cells is not restricted to the expression of specific HLA-DR alleles.

Next, Stohl et al. [55] used nonirradiated T cells and eight different SEs (SEA, SEB, SEC_1, SEC_2, SEC_3, SED, SEE and TSST-1) to study the effects on Ig secretion. These superantigens, typically at concentrations 10^2-10^4 times lower (100 vs. 0.01–1 ng/ml) than required for T cell proliferation, allowed

unmanipulated (nonirradiated) peripheral blood or tonsil T cells to drive polyclonal B cell proliferation and Ig secretion in culture, but demanded an adequate number of B cells to serve as (DR+) accessory cells. They found that the mixture of purified T cells and B cells in ratios between 5:2 and 1:3 could replace PBMC cultures, in which the T cell:DR+ cell ratio is 3:1, and tonsil cells, in which it is 1:3. They observed that monocytes supplement the DR+ cells in PBMCs, and noted that, although tonsil cells are deficient in monocytes, the T cell:B cell ratio, 1:3, can compensate. Stohl et al. [55] also demonstrated that antibodies against the costimulatory molecules, CD28 (CTLA-4) on T cells and B7-like molecules on B cells, inhibited superantigen-induced Ig secretion to a greater extent than that by anti-CD3-activated T cells and B cells. These observations suggested that there might be a window of opportunity in tissues, when the superantigen dosage and cellular composition are 'right' for T cell superantigens to induce the differentiation of B cells into Ig-secreting cells. They further demonstrated the importance of the costimulatory molecules CD28 (CTLA-4) and B7 in superantigen-induced B cell differentiation (fig. 1c).

Lester et al. [56] showed that TSST-1 at 1 pg/ml inhibited IL-4-stimulated synthesis of IgE, but not of IgG, in PBMCs from AD patients and normal donors. Inhibition of IL-4-induced IgE production was associated with the induction of IFN-γ secretion by TSST-1. This inhibitory effect was blocked by a neutralizing antibody against IFN-γ (normal PBMC) or IFN-α (AD PBMC). (In normal blood, IFN-γ exceeds IFN-α concentrations, whereas in AD blood, production of IFN-γ by T cells is inhibited, but the production of IFN-α by monocytes is increased.) This provided the first clue that TSST-1 (at 1 pg/ml) might inhibit class switching to IgE by enhancing the synthesis of IFN-γ and IFN-α.

Hofer et al. [57] demonstrated the antagonistic effects on IgE synthesis at the extremes of TSST-1 concentration between 0.01 and 1,000 pg/ml. Incubation of PBMCs with 1,000 pg/ml inhibited, while 0.01 pg/ml enhanced IgE synthesis in vitro. In the presence of a blocking IFN-γ antibody, but not otherwise, TSST-1 at 1 pg/ml stimulated IgE synthesis. IgG and IgA also rose after TSST-1 stimulation. B cells underwent apoptosis at the higher TSST-1 concentrations. The addition of IFN-γ to cultures of PBMCs with TSST-1 at 1 pg/ml had the same effect as culturing PBMCs with TSST-1 at 1,000 pg/ml. Culturing purified B cells with IFN-γ did not induce apoptosis of B cells, indicating that the B cells need to be activated in some way by TSST-1 if they are to undergo IFN-γ-mediated cell death.

Jabara and Geha [58] reported that incubation of the PBMCs at TSST-1 concentrations of 1–10 ng/ml inhibited IL-4-induced IgE synthesis in a dose-dependent manner, and that anti-IFN-γ suppressed this effect. Strong stimulation of IgE synthesis with TSST-1 and IL-4 was also reproduced in mixtures of

purified T cells and B cells in ratios of 0.05:10 to 4:10 (thus minimizing IFN-γ secretion). Jabara and Geha [58] showed that incubation of the T cells with TSST-1 stimulates the expression of CD40L on the subset of T cells expressing the cognate Vβ2. Stimulation of CD40L expression on T cells suggests that TSST-1 acts by elevating CSR, rather than (or in addition to) provoking proliferation of preswitched IgE-expressing B cells. Accordingly, a neutralizing anti-CD40L mAb blocked the stimulation of IgE synthesis. When T cells were incubated with TSST-1, washed to remove superantigen, fixed with paraformaldehyde, and incubated with B cells in the absence of TSST-1, the B cells still produced IgE, implying that the exogenous IL-4 and CD40L expression on the fixed T cells is sufficient to induce class switching to IgE. Jabara and Geha [58] could also conclude that signalling through MHC II proteins is unnecessary for the effects of TSST-1 on class switching to IgE. Thus, the signals emanate from the costimulatory activity of CD40 and CD28 on T cells, when they interact with CD40L and B7 on the B cells. Jabara and Geha [58] proposed that similar events could take place in tissues containing appropriate ratios of T cells to B cells.

Jirapongsananuruk et al. [51] followed up the observations on the upregulation of B7.2 in superantigen-stimulated PBMCs. They reported that the expression of B7.2 was higher on B cells from patients with AD than from normal subjects or patients with psoriasis (a predominantly Th1-type skin disease). Total serum IgE concentrations correlated with B7.2 expression by B cells from AD patients and the synthesis of IgE by purified B7.2+ B cells in vitro. Anti-human B7.2 but not B7.1 suppressed IgE production by PBMCs that had been stimulated with IL-4 and anti-CD40. The expression of IL-4 receptor and CD23, both associated with augmentation of IgE responses, was higher on B7.2+ than B7.1+ B cells. These data are consistent with the view that the costimulatory interaction between CTLA-4 on Th2 cells and B7.2 on B cells enhances cross-talk between the cells, leading to an increase in IL-4 secretion and CD40L expression by the Th2 cells, and an increase in B cell survival, proliferation, class switching to IgE, differentiation into plasma cells, and IgE production.

Hofer et al. [59] questioned whether allergen-specific IgE responses, as well as total IgE responses, are enhanced by the incubation of PBMCs with TSST-1. They isolated PBMCs from atopic patients during and outside the pollen allergen season and stimulated them with TSST-1 at 0.01 or 1 pg/ml. Out of season, TSST-1 increased total IgE production only in the presence of exogenous IL-4, whereas during the pollen season endogenous IL-4 (released by Th2 cells and mast cells) was apparently sufficient to stimulate IgE production. The increase in IL-4 was accompanied by a decrease in IFN-γ during the pollen season. No seasonal effect was observed for the stimulation of IgG production

in PBMCs incubated with TSST-1, as both IL-4 and INF-γ stimulate switching to IgG in B cells, whereas IL-4 or IL-13 are required for IgE [60–62].

To investigate the link between TSST-1 and the production of allergen-specific IgE, Hofer et al. [59] isolated PBMCs from cat-allergic patients, who were either living with cats and currently symptomatic, or asymptomatic without recent exposure to cats. TSST-1 enhanced cat allergen-specific IgE only in PBMCs from the cat-allergic patients with symptoms. The increase in allergen-specific IgE pertained only to the allergen to which the patients were sensitized. Similar conclusions emerged from the synthesis of IgE specific for grass and ragweed pollen. TSST-1 did not cause the production of allergen-specific IgE in PBMCs from normal donors. Hofer et al. [59] proposed the model for the effects of TSST-1 on allergen-specific IgE production depicted in figure 1c.

Tumang et al. [63] used a mouse model to explore whether *S. aureus* T cell superantigens promote Th-cell-dependent, antigen-specific antibody responses. Although directed towards an understanding of superantigen effects in autoimmunity and graft versus host disease, the outcome is relevant here because of the authors' success in extending the work from an in vitro to an in vivo system. They investigated the potential of TSST-1, as well as a *Mycoplasma-arthritidis*-derived superantigen, to support Th-cell-dependent, antigen-specific antibody responses in BALB/c murine T cell clones specific for these antigens. They first generated the TSST-1- or *Mycoplasma-arthritidis*-specific Vβ-specific T cell clones. After coculture of irradiated T cell clones with syngeneic B cells, they observed B cell proliferation and polyclonal IgM and IgG production. In the presence of sheep red blood cells, synthesis of sheep red blood cell-specific antibodies supervened. This led to the conclusion that the TSST-1 cognate Th cells could provide selective help for proliferation and/or differentiation of antigen-specific B cells in the presence of the antigen. The same study revealed that in vivo administration of TSST-1 to nude mice results in superantigen binding to B cells, rendering these cells effective stimulators of, and targets for, the superantigen-reactive Th cells. This affords support for the relevance of their observations in vitro.

A general point is that workers in the fields of autoimmunity and allergy have reached similar conclusions concerning the effects of SEs and other superantigens. On both sides of the Th1 (autoimmunity)/Th2 (allergy) divide, superantigens appear to assist the proliferation of B cells producing pathogenic antibodies of one class (IgM or IgG) or the other (IgE). This scenario is depicted in figure 3. In either (Th1 or Th2) environment, superantigens might stimulate polyclonal B cell proliferation. Among the stimulated B cells in predisposed individuals might be rare self-antigen- or allergen-specific B cells. A threshold number of such cells could allow the progression of disease. In the presence of self-antigen or allergen, these B cells might proliferate and differentiate

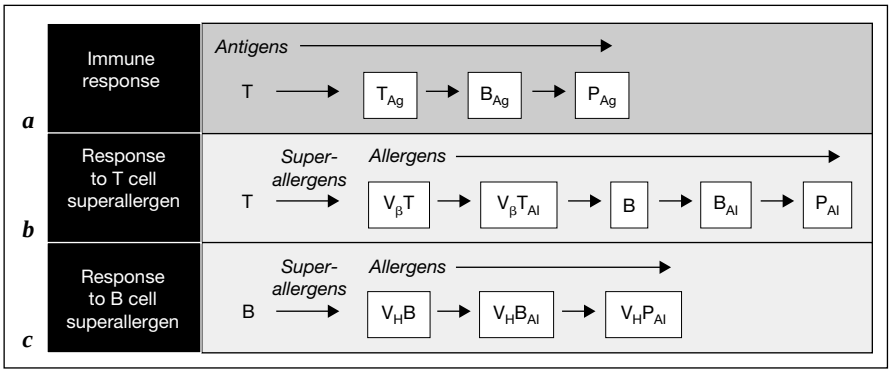

Fig. 3. *S. aureus* superallergens can trigger or exacerbate allergic responses. ***a*** Antigen-activated Th cells specific for conventional antigens (T_{Ag}) interact with antigen-activated cognate B cells (B_{Ag}) and stimulate B_{Ag} proliferation and differentiation into plasma cells secreting antibodies directed against the antigen (P_{Ag}). ***b*** A T cell superallergen can induce the polyclonal stimulation of a subset of Th cells (VβT) and polyclonal B cell proliferation. In the presence of allergen, the stimulated VβT specific for allergen (T_{Al}) can help the allergen-specific B cells (B_{Al}) interspersed among the stimulated B cells ($V\beta B_{Al}$) that differentiate into plasma cells secreting antibodies directed against the allergen (P_{Al}). ***c*** A B cell superallergen can induce the polyclonal stimulation of a subset of B cells ($V_H B$). Then allergens can further stimulate the allergen-specific B cells ($V_H B_{Al}$) amongst the stimulated $V_H B$ that differentiate into plasma cells secreting antibodies directed against the allergen ($V_H P_{Al}$).

into long-lived plasma cells and secrete potentially pathogenic antibodies [64]. Depending on the Th cell type preponderating in the tissue (synovial joint/mucosa), the antibodies might be IgM and IgG (autoimmunity) or IgE (allergy).

Effects of Topical Application of Superantigens in vivo

Strange et al. [65] demonstrated that SEB induces eczema when applied to intact healthy and atopic skin. Skov et al. [66] sought to discover the mechanism of this effect. Inquiring whether the topical application of SEB to skin leads to the accumulation of T cells in the skin by a superantigen-mediated mechanism, they observed an increase in skin thickness and increased number of T cells in the skin peaking at 72 h. Application of SEB led to selective accumulation of the expected Vβ12+ and Vβ17+ T cells at the site, but not in untreated skin or PBMCs from the treated subjects. Thus, the enterotoxin provokes only local changes in the skin on this timescale.

Using a similar approach in a mouse model, Laouini et al. [67] observed that epicutaneous administration of SEB induced a systemic SEB-specific IgE antibody response and allergic skin inflammation. Savinko et al. [68] found that in ovalbumin-sensitized mice, local administration of SEB induced both SEB-specific IgE synthesis and elevated production of ovalbumin-specific IgE. Both studies concurred in finding allergic skin inflammation evinced by the presence of increased numbers of Vβ8+ Th2 cells and eosinophils, and expression of Th2-type cytokines and chemokines.

Local effects of SEB inhalation in the bronchial mucosa cannot be tested experimentally in humans, but have been in mice [69–71]. Low doses of SEB were sufficient to cause some of the characteristic signs (e.g. eosinophilia) and symptoms (bronchial hypersensitivity and airway hyperresponsiveness) of asthma.

Additional Pathogenic Mechanisms of *S. aureus* Enterotoxins

Basophil Activation

S. aureus cultured from skin lesions of AD patients released SEA, SEB, SEC, SED and TSST-1, and IgE antibodies against the SEs were also detected in the circulation of half of the subjects. SEA, SEB and TSST-1 triggered histamine release when incubated with basophils isolated from AD patients whose serum contained the corresponding anti-SE IgEs, but not from basophils isolated from normal controls [72]. When IgE was stripped from the basophils of the controls, which were then sensitized with serum from the AD patients, the SEs triggered histamine release. The effector functions of the anti-SE IgEs reveal that the anti-SE IgEs are unlikely to be a mere epiphenomenon, as some authors have asserted, but could play a role in the pathophysiology of AD. Local histamine release, which may well arise partly from the sensitization of basophils and mast cells by anti-SE IgEs (especially in the absence of allergen provocation), would exacerbate the itch-scratch cycle in AD and lead to the continuing production of proinflammatory cytokines.

T Regulatory Cell Inhibition

CD4+CD25+ T regulatory cells (Tregs) play an important part in controlling inflammatory responses. Ou et al. [73] incubated PBMCs with anti-CD3, measured cell proliferation, and phenotyped the resulting cell population. They confirmed the identity of CD4+CD25+ cells as Tregs by real-time PCR for FoxP3 mRNA, a distinctive marker for these cells. They observed an increase in Tregs in patients with AD relative to control subjects. Consistent with the known properties of Tregs, the CD4+CD25+ cells were anergic to anti-CD3

stimulation, and, when mixed with CD4CD25-T cells, suppressed anti-CD3 stimulation of these cells. Significantly, however, after SEB stimulation, CD4+ CD25+ cells were no longer anergic, nor could they suppress SEB stimulation of CD4+CD25− cells. This represents a hitherto unappreciated aspect of pathogenic SE activity.

Confounding Steroid Therapies for Asthma

Glucocorticoids (GCs) are the mainstay of anti-inflammatory therapy, but some 25% of asthmatic patients are resistant to GC therapy, presenting a serious problem in the management of chronic asthma [74–77]. A series of investigations based on knowledge of the mechanism of steroid action, reviewed below, implicates SEs in steroid resistance.

A major role in steroid insensitivity has been assigned to activated T cells, which can be detected in the bronchoalveolar lavage (BAL) and PBMCs of patients with asthma [78, 79]. Studies on PBMCs from patients with GC-insensitive asthma revealed that GCs fail to inhibit mitogen-induced T cell proliferation and cytokine secretion in vitro [80, 81]. In addition, T cells in peripheral blood of GC-insensitive, but not GC-sensitive, asthmatics are persistently activated, despite high doses of GC therapy [82].

GCs act by binding to a cytoplasmic glucocorticoid receptor (GCR), which then translocates to the nucleus as a transcription factor. Sher et al. [83] found that PBMCs from the majority of patients with GC-insensitive asthma had a reversible defect in GCR ligand binding affinity, which could be sustained in vitro by addition of IL-2 and IL-4. In vitro incubation of normal blood T cells with the combination of IL-2 and IL-4 reduced their GCR ligand binding affinity to the level seen in GC-insensitive asthma [84]. Analysis of bronchial biopsies and cells in BAL has revealed that airway T cells in GC-insensitive asthmatics have significantly higher levels of IL-2 and IL-4 gene expression than those of GC-responsive patients [85].

Against this background, Adcock et al. [86] reported that GCRs in PBMCs from patients with GC-insensitive asthma have a decreased ability to bind their DNA recognition sequences (GC response elements or GRE). Leung et al. [87] confirmed that PBMCs from the majority of patients with GC-insensitive asthma have a reversible defect in GCR ligand binding affinity. They found that this defect could be explained by the predominance of an alternative splice variant of the GCR, GCRβ. Sousa et al. [88] compared skin biopsies from GC-sensitive and GC-insensitive asthmatics, before and after a course of prednisolone, and showed that the number of cells expressing GCRβ and the ratio of GCRβ: GCRα were elevated in the patients who were GC-insensitive. Oral prednisolone

treatment led to a decrease in the number of cells expressing GCRα, but not GCRβ, in the subjects who were GC-sensitive but not in those who were resistant. They suggested that the increased expression of GCRβ might be a critical mechanism for conferring GC resistance. Consistent with this hypothesis, increased expression of GCRβ was detected in airway cells in GC-insensitive asthma [89] and in fatal asthma [90].

The two GCR isoforms, GCRα and GCRβ, have three domains, an N-terminal immunoregulatory domain, a central DNA binding domain and a C-terminal ligand binding domain. Both isoforms are identical from amino acids 1–727, but differential splicing in exon 9 replaces the most C-terminal 50 of GCRα with 15 different amino acids in GCRβ, thereby reducing the DNA binding affinity [91]. Airway cells from GC-insensitive patients contain a higher proportion (~20%) of the inactive GCRβ form than GC-sensitive patients (~10%) or normal controls (~5%) [89]. Transfection of a HepG2 cell line with an expression vector for GCRβ reproduced the GC resistance exhibited by PBMCs isolated from the GC-insensitive patients [87]. The transactivation and repression activities of the various N-terminal isoforms of GCRα and GCRβ, which are regulated at the level of translation [92], and the posttranslational modifications of the hormone receptors [93] have not yet been studied in the context of allergic disease.

Monomeric GCR in the cytoplasm exists as a complex with heat shock protein. Upon GC binding, GCR dissociates from this complex, dimerizes and translocates to the nucleus where it binds to response elements (GRE) in the regulatory regions of the target genes [94]. It was conjectured that GCRβ might, by forming GCRα-GCRβ heterodimers, interfere with the normal activity of the GCRα homodimers, thus acting as dominant negative inhibitor. GCRα also interacts with other important transcription factors (AP-1, NFκB), which carry GCRα to their response elements in genes that encode inflammatory proteins; in this mode of activity, GCRα appears to function as a corepressor [95]. It is not known which of these two kinds of activity GCRβ perturbs, direct DNA binding to GRE or indirect binding to AP-1 or NFκB sites, if it indeed affects either of them. It may merely sequester the transcription factors and so prevent DNA binding. In fact, very little appears to be known concerning the exact mechanisms by which the GCRα homodimer exerts its activity as a repressor of gene activation. It is likely nonetheless that GCRβ functions as a dominant negative inhibitor of GCRα, thereby allowing cytokine stimulation of the genes for inflammatory proteins, which GCRα would normally be able to repress.

Colonization by *S. aureus* in AD and respiratory infections in asthma is associated with both worsening of clinical symptoms and increased requirements of GCs [96]. Hauk et al. [96] tested the hypothesis that superantigens may induce GCRβ and thereby cause GC insensitivity in asthma. They demonstrated

that subjects with poorly controlled asthma had a significantly higher expression of Vβ8+ T cells in BAL fluid than those with well-controlled asthma or normal controls. Increased Vβ8+ T cells were present in both CD4+ and CD8+ populations, suggesting general selection for Vβ8+ T cells due to superantigen activity. The Vβ8+ upregulation in BAL was significantly higher than that of the PBMCs of poorly controlled asthmatics. About half of the subjects with poorly controlled asthma were taking inhaled GCs, but the upregulation of their Vβ8+ T cells in BAL did not differ significantly from the others, indicating that they were resistant to GC therapy.

While investigating signalling pathways that could participate in superantigen-induced GC resistance, Li et al. [97] observed that PBMCs stimulated with SEB, but not anti-CD23, induced GC-insensitive T cells. However, the purified T cells were susceptible to GC inhibition by both agents. The results implied that signals on APC might act in concert with the TCR to cause steroid resistance. Blockade of CD40-CD40L interaction had no effect on the GC resistance; but CD28 costimulation with TCR activation induced GC resistance in the purified T cells in a dose-dependent manner. This included a more rapid and sustained phosphorylation of the signalling kinase ERK and treatment with a specific inhibitor of this kinase restored the response to GCs. They showed that ERK was able to phosphorylate GCRα directly in vitro and that SEB-induced GC resistance was associated with abrogation of GCRα nuclear translocation, and was reversible by the ERK inhibitor. The results demonstrated that SEB-induced GC resistance is associated with the phosphorylation of GCRα and inhibition of GC-induced GCRα nuclear translocation. But it is unclear whether this, or the induction of GCRβ by GC is the proximal cause of the defect in nuclear translocation.

Goleva et al. [74] used a range of approaches to determine whether GCRβ interferes with the translocation of GCRα to the cell nucleus. They transfected mouse cells with an expression vector for GCRβ and employed antibodies directed against GCRα and GCRβ for both immunohistochemistry and Western blotting of cytoplasmic and nuclear cell fractions, to visualize the distribution of the two isoforms in the cells. The majority of GCRβ was identified in the cytoplasm and the Western blots showed that GCRβ prevented translocation of GCRα into the nucleus in response to steroids. For confirmation, they used real-time PCR to examine transcription of a gene encoding an early marker of steroid activity. It emerged that GCRα transactivation of the marker gene was proportionately reduced by GCRβ expression, implying that the elevated cytoplasmic GCRβ inhibits GCRα translocation to the nucleus and transactivation of the marker gene.

Goleva et al. [74] then transfected BAL macrophages from patients with GC-insensitive asthma with GCRβ siRNA. The cells were treated with the

steroid analogue dexamethasone and a steroid marker gene expression was monitored. GCRβ mRNA was shown to be suppressed relative to cells transfected with nonsilencing control siRNA, and the effect was shown to be GCRβ specific, since GCRα expression was unaffected. There was a significant enhancement of dexamethasone-induced marker mRNA production when GCRβ was suppressed. GCRβ expression in both cytoplasmic and nuclear compartments fell, so that it could not be established in which compartment(s) GCRβ exerted its activity.

To study possible mechanisms by which superantigens could contribute to poor disease control, Hauk et al. [98] incubated PBMCs with SEB, SEE and TSST-1 and found that this prevented the ability of dexamethasone to inhibit PHA-induced PBMC proliferation. This indicated that these SEs can induce GC resistance. Stimulation of normal PBMCs with SEB induced an increase in GCRβ expression, compared to PHA. The conclusion was that superantigens may contribute to GC insensitivity through induction of GCRβ.

Fakhri et al. [99] incubated biopsies from ragweed-sensitive patients with ragweed allergen in the presence or absence of both SEB and dexamethasone. They examined the expression of GCRβ by immunocytochemistry and that of IL-2 and IL-4 mRNA by in situ hybridization. SEB induced an increase in the expression of GCRβ in atopic tissue and to a lesser extent in nonatopic tissue. The most significant effect came in response to the combination of SEB and ragweed allergen: stimulation with ragweed alone induced IL-4, and stimulation with SEB alone induced IL-2 mRNA. Incubation with both ragweed allergen and SEB induced both cytokines. The increase in IL-4 mRNA was reduced by the addition of dexamethasone to atopic tissue stimulated with ragweed alone, but not to tissue stimulated by both ragweed and SEB. These authors concluded that superantigens induce steroid resistance in atopic nasal tissue by upregulating the expression of GCRβ. These results emphasize that the upregulation of GCRβ is a local event, associated with the coexpression of IL-2 and IL-4 mRNA.

Local Class Switching to IgE

Before they are competent to secrete IgE antibodies with high affinity for allergens, B cells must undergo affinity maturation and class switching to IgE, as described above, in the germinal centers of lymphoid tissue. They must also undergo differentiation into Ig-secreting plasma cells, either in lymphoid tissue or following the migration of memory cells into inflamed tissues. Alternatively, as recent studies have revealed, all three stages in B cell development occur locally at sites of allergic inflammation [2].

The respiratory tract mucosa affords a favorable environment for class switching to IgE. Activated Th2 cells and mast cells in the nasal mucosa in allergic rhinitis and in the bronchial mucosa in asthma collectively secrete IL-4 and IL-13, as well as additional proinflammatory cytokines (including IL-3, IL-5, IL-9, GM-CSF, TNF-α), chemokines and other mediators (lipids, proteases and histamine). These exert regional effects in the mucosa, inducing the recruitment of inflammatory cells from the circulation and activation of these cells in situ [47, 100]. The activated T cells and mast cells express IL-4, IL-13 and CD40L [101, 102], the signals needed for class switching to IgE.

εGLT is required for switching to IgE (fig. 2) and ε-chain mRNA for IgE protein synthesis. To examine the possibility of local class switching to IgE, Durham et al. [103] and Cameron et al. [104] employed in situ hybridization to detect εGLT and ε-chain mRNA in nasal biopsies from allergic rhinitis patients. Positive results for both markers were obtained from patients exposed to allergen, either during the pollen season or 24 h after local allergen challenge out of season. Only ε-chain mRNA was detected when the patients were not exposed to allergen. εGLT was not expressed in the nasal mucosa of nonallergic controls; ε-chain mRNA was expressed, but at lower levels compared with the allergic rhinitis patients. Increases in εGLT expression were correlated with those of IL-4 and IL-13 mRNA in the biopsies, consistent with the known effect of allergen provocation on local expression of these cytokines and their ability to stimulate εGLT expression. Cameron et al. [105] also incubated nasal biopsies ex vivo and demonstrated that εGLT expression was inhibited by anti-IL-4. ε-Chain mRNA-expressing cells were also more frequent in the bronchial biopsies from (both atopic and nonatopic) asthmatics than in those from control subjects [106, 107].

To examine CSR (the irreversible step in class switching, subsequent to germline gene transcription) directly, Cameron et al. [108] used PCR to detect circular DNA, while Takhar et al. [109] detected the switch CTs by RT-PCR in nasal biopsies from allergic rhinitis patients. As expected, these markers occurred predominantly in biopsies from the allergic patients, and much less frequently in control subjects. The results demonstrated recombination from IgM, and from the three IgG subclasses tested (IgG1, IgG3 and IgG4) to IgE. Crucially, it could be shown that incubation of the biopsies from allergic patients with the allergen to which they were sensitized ex vivo induced the appearance of the same markers. This excluded the possibility that CSR occurred in the local lymph nodes or in extrinsic lymphoid tissue, followed by rapid migration of the B cells into the nasal mucosa. These results established the principle of local class switching to IgE (reviewed in Gould et al. [2] and Fiset et al. [110]).

The enzyme AID was shown to be required for CSR (and SHM) in B cells, but was reported to be restricted to B cells in lymphoid follicles [50]. However, as required for local CSR, Coker et al. [32] and Takhar et al. [109] detected AID mRNA in the nasal mucosa of allergic rhinitis patients. Unlike εGLT and εCT, however, AID mRNA was expressed in the mucosal tissues from allergic rhinitis patients in the absence of allergens, but not in those of controls. The presence of AID mRNA may reflect a fundamental abnormality that predisposes some individuals to disease in these target organs.

After CSR, a further step leads to the expression of IgE, for the B cells must undergo differentiation into IgE-secreting plasma cells. The co-localization of ε-chain mRNA with B-cell-specific markers by dual immunohistochemistry and in situ hybridization in the nasal biopsies of rhinitics and bronchial biopsies from asthmatics indicates that this is highly likely [103–106], and the same conclusion also emerges from assays for IgE protein and IgE protein synthesis.

Kleinjan et al. [111] used dual immunohistochemistry for B cell and plasma cell markers and IgE to count IgE-expressing cells in the nasal mucosa of allergic rhinitis patients. Some 4% of the B cells and 12–19% of the plasma cells expressed IgE. The frequency of IgE-expressing B cells in the circulation is normally around 0.01% [112], that is to say three orders of magnitude lower. Surprisingly, even the nasal mucosa of the normal subjects displayed a relatively high proportion (1%) of IgE+ B cells, which is still two orders of magnitude greater than that in the peripheral blood B cell population. Yet no IgE+ plasma cells could be detected, suggesting a failure of the B cells to undergo the final stage of class switching, differentiation into IgE-secreting plasma cells, in normal subjects. Kleinjan et al. [111] also used dual immunohistochemistry to measure the proportion of plasma cells with bound allergen. The similarity to the proportion expressing IgE raised the possibility that the two populations, allergen-specific and IgE-expressing plasma cells, might overlap, that is to say, embrace a sizeable fraction of plasma cells expressing allergen-specific IgE.

To establish whether IgE synthesis occurs in the nasal mucosa in allergic rhinitis, Smurthwaite et al. [113] incubated nasal biopsies from allergic rhinitis patients ex vivo and measured the production of IgE by ELISA and by radioactive amino acid incorporation into IgE. IgE levels produced by nonallergic biopsies were significantly lower than those from the allergic subjects. The biopsies from the allergic patients generated a surprisingly large proportion (up to 50%) of allergen-specific IgE, invariably higher than that in serum of the same individual. Although the biopsies produced more IgG than IgE ex vivo, no allergen-specific IgG was detected. This might mean that all of the allergen-specific IgG-expressing B cells had switched to IgE or that the population of

allergen-specific IgE-expressing B cells was selectively enlarged before differentiating into plasma cells.

Local Somatic Hypermutation

It is unlikely that IgE antibodies with extremely low affinity for allergen would have been detected in the experiments of Smurthwaite et al. [113]. Therefore, the B cells could have undergone affinity maturation, involving SHM and clonal expansion before or after migration into the tissue. But the expression of AID in the nasal mucosa makes this the more likely site.

Evidence for local SHM was first detected in a bronchial biopsy from a patient with asthma. Snow et al. [114] cloned and sequenced Vε cDNAs obtained by RT-PCR from a bronchial biopsy. There were many identical sequences in these clones, and the majority contained multiple somatic mutations, identified by comparison with the nearest germline V_H gene sequence. Some of the sequences could be arranged in family trees, characterized by unique and shared mutations relative to the germline V_H gene sequence, which represents their common B cell progenitor (identified by the VDJ junctional sequence; see above). Vε clonal families in the bronchial mucosa suggest that local SHM and clonal expansion occurred in the tissue, given the low probability that sister clones would all have migrated from a remote location to the region of tissue sampled in the biopsy. However, Vε clonal families were also found in the spleen of an asthmatic patient [115] and in the peripheral blood cells of several patients [116, 117]. This casts doubt on the importance of local SHM, and even on the conclusion that it actually occurs.

The authors reported another curiosity, the overexpression of the minor V_H5 family in IgE in asthma. The V_H5 family is one of the smallest families in the human V_H germline gene repertoire, encompassing only 2 of the 51 functional V_H germline genes. It is normally proportionately expressed in about 3% of peripheral blood B cells [39]. The proportion in the B cells from asthmatic patients (counting unique clones and clonal families only once) was 30%, a tenfold overexpression, compared to the normal repertoire. These sequences also exhibited other unusual properties, including a shift in the proportion of somatic mutations causing amino acid substitutions from CDRs to FRs, and the location of 'nonintrinsic' mutations (those that occur in sequences deviating from the consensus sequences for somatic mutations in V_H genes). These observations led to the conclusion of a possible action of a B cell superantigen, which would be consistent with the V_H5 bias, the R/S values (ratio of replacement to silent nucleotide substitutions) in FRs and CDRs and the location of the nonintrinsic mutations.

Remarkably similar results were reported in studies of the nasal mucosa in allergic rhinitis patients [32, 118]. Three Vε clonal families were observed in the nasal biopsies from 2 out of 6 allergic rhinitis patients, but, unlike in the asthma patients [114–117], none were found in their PBMCs. One of the clonal families was a Vε3 family and two (from different patients) were Vε5 families. An overall Vε5 bias (30% vs. the normal 3%) was also observed in the IgE, and a smaller bias to V_H5 in the IgA from the biopsies of allergic rhinitics [118]. The same peculiarities in the R/S values and the distribution of nonintrinsic mutations were also found in the Vε and Vα sequences in asthma. These general features of V_H sequences in the respiratory tract mucosa are strikingly similar to the effects that would be engendered by the local activity of a B cell superantigen. These of course would not be limited to a single antibody class, as observed.

A strong bias towards V_H5 as well as the peculiarities in V_H5 sequences found in asthma and allergic rhinitis were also seen in one study of the PBMCs in AD patients [119]. A general tendency towards a V_H5 expression in B cells in allergic disease may therefore be inferred. However, since some of these observations were made on PBMCs, the question arises whether the selection of these B cells by the putative superantigen occurred in the target organ, followed by their escape into the circulation, where they were detected, or whether the B cells in the target organ were recruited from the circulation after selection at another anatomical site. The original rationale hinged on the improbability that sister clones could have migrated to, and proliferated at the same small region of the tissue. To obtain an idea of the volume of the affected region, Coker et al. [118] examined the Vε sequences in two adjacent 2 mm^3 biopsies. In biopsy A, 11/11 unrelated sequences were Vε5. In biopsy B, 0/4 were Vε5. Similar results were obtained when the Vα sequences were analyzed. In biopsy A, 2/12 of the sequences were Vα5, neither of them related to each other or to the Vε5 sequences in the same biopsy. In biopsy B, 0/10 of the sequences were Vα5. Taken together, in IgE and IgA, 13/23 in biopsy A and 0/14 in biopsy B were V_H5. Thus, factors that induce the V_H5 bias appear to be restricted to a volume of less than 2 mm^3.

No B cell superantigen specific for V_H5 has yet been discovered. SpA and SEA select the largest V_H3 family, whereas SED selects the second largest V_H4 family of V_H genes. However, *S. aureus* colonization of mucosal tissues is very prevalent and significantly increased in the target organs in allergic disorders and airway disease. *S. aureus* produces a large number of SEs, most of which have not yet been tested for B cell superantigen activity. One or more of these SEs or other superantigens could be responsible for the observed V_H5 bias and other peculiarities seen in the V_H5 sequences in the target organs of allergic individuals. The activity of the putative B cell superantigen may occur in a

heterogeneous pattern in mucosal tissues, according to the local concentration (dosage) of the superantigen and the relative proportions of different cells in the affected region or other microenvironmental factors, which would be consistent with the unequal distribution of V_H5 sequences seen in the nasal mucosa of the allergic rhinitis patients [118].

Summary and Conclusions

We have seen how T cell superantigens can increase the concentrations of B cells that elevate allergen-specific (or autoantigen-specific) antibodies to dangerous levels. We have also described the interaction between B cell superantigens and Ig BCRs, which could leave room for the binding of allergens (or autoantigens), so as to allow synergistic stimulation of B cell proliferation and the production of pathogenic antibodies. We have reviewed the evidence that SEs exert many of their effects locally in the target organs in allergic disorders and airway disease, and that they induce class switching to IgE and the production of allergen-specific IgE antibodies. In chronic allergic inflammation, B cells undergo local proliferation, SHM and class switching to IgE, and they differentiate into allergen-specific IgE antibody-secreting plasma cells. One may expect that, at least in some cases, this abnormality in IgE regulation will represent the culmination of many of the other pathological processes triggered by superantigens, produced locally by *S. aureus* (and other superantigen-generating bacteria) in the target organs in allergic disorders and airway disease.

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Marone G (ed): Superantigens and Superallergens.
Chem Immunol Allergy. Basel, Karger, 2007, vol 93, pp 137–160

Superantigen-Induced Regulatory T Cells in vivo

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Abstract

Bacterial and viral superantigens (SAgs) can induce the activation of a large fraction of the T cells in an individual. The activated T cells divide and produce a strong cytokine response. Subsequently, the majority of the expanded T cells are deleted and the remaining SAg-reactive cells will be anergic and tolerant to SAg exposure. The anergic population is heterogeneous and includes both T cells with induced nonresponsiveness to the SAg and SAg-specific regulatory T cells (Tregs). Both CD4+CD25− and CD4+CD25+ Tregs are generated in SAg-induced responses. The Tregs suppress T cell activation and produce the immunosuppressive cytokines IL-10 and TGF-β_1, thereby contributing to the SAg-specific tolerance. The similarities of immune responses induced by SAgs and conventional peptide antigens administered in the absence of adjuvants will be discussed. It is proposed that the antigen-specific tolerance induced in both situations is the consequence of T cell activation mediated by immature dendritic cells.

T cells respond to foreign protein antigens presented on major histocompatibility complex (MHC) molecules. The antigens are first processed to short peptides inside the cell that expresses the presenting MHC molecules – the antigen-presenting cell (APC). Both proteins produced inside the cell and proteins acquired from the outside by endocytosis are processed. The peptides so generated bind to a cleft formed by the amino-terminal part of the MHC proteins [1]. This part of the MHC proteins is highly polymorphic and the various allelic variants of a certain MHC protein give rise to structurally different peptide-binding clefts. Consequently, different peptides bind to the various allelic variants of an MHC molecule [2].

T cells recognize MHC/peptide complexes via their antigen-specific T cell receptors (TCRs). The TCR molecule binds to amino acid residues of both the

MHC molecule and the peptide, which are exposed on the MHC/peptide complex (reviewed in Jorgensen et al. [3]). In an individual exposed to a certain MHC/peptide complex for the first time, the fraction of reacting T cells will usually be low – in the order of $1/10^4 - 1/10^5$.

Certain protein antigens of microbial origin, denoted superantigens (SAgs), activate a large fraction of the T cell repertoire – in the order of several percent of the T cells. The most commonly studied SAgs are the staphylococcal enterotoxins and the retroviral SAgs encoded by mouse mammary tumor viruses (MMTVs). The molecular basis for the high frequency of T cells reacting to SAgs is that these proteins, without the need for previous processing, bind both the MHC [4] and TCR molecules creating a cross-linking signal [5]. The SAgs bind to relatively conserved regions of MHC II molecules therefore allowing for binding to several different allelic forms of the molecules. On the T cell, SAgs bind to sites on the Vβ chain of the TCR, therefore inducing the activation of T cells expressing those particular TCR chains (reviewed in Petersson et al. [6]). Thus, the consequence of SAg exposure is the activation of a large fraction of the T cells in an individual.

The Superantigen-Induced Immune Response

In vivo exposure to SAgs induces activation of both CD4+ and CD8+ T cells expressing certain Vβ chains in their TCRs. The activated T cells divide and produce cytokines, similarly to T cells induced by conventional protein antigens (fig. 1). Subsequently, the majority of the expanding T cells will be deleted, but the extent of the deletion is often more extensive than that seen in responses to conventional antigens, leading in some cases to a reduction below preimmune cell numbers. In contrast to the response to immunization with conventional antigens together with adjuvants, the remaining SAg-reactive T cells will be anergic rather than memory cells, as they do not proliferate when reexposed to the SAg in vitro. The resulting state of tolerance is thought to be beneficial both to the host, as it limits cytokine-induced inflammation, and to the microbe because of the reduced immune response.

Cytokine Production

The bacterial SAgs (bSAgs) staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin B (SEB) rapidly induce massive cytokine production in vivo that can cause toxic shock or death [7, 8]. Thus, high serum levels of IL-2 and TNF-α are detected within 1 h after injection into mice [9–11].

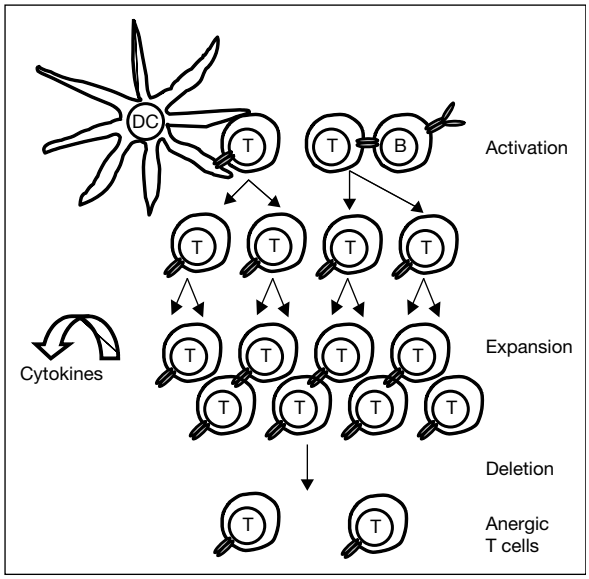

Fig. 1. SAg-induced CD4+ T cell response. DCs and B cells present the SAgs to CD4+ T cells, inducing T cell activation, cytokine production and proliferation. A majority of the expanding cells will subsequently be deleted by induction of apoptosis and the remaining CD4+ T cells will be anergic upon subsequent exposure to the immunizing SAg.

Subsequently, serum levels of IL-1, IL-6 and IFN-γ increase. CD4+ T cells are the main producers of IL-2 and TNF-α, while CD8+ T cells are the main producers of IFN-γ [11, 12]. In contrast, IL-4, IL-5 and IL-10 are produced only at low levels in the acute SAg-induced response [11]. After repeated exposure to bSAgs, the serum levels of IL-2 and IFN-γ are reduced while there is an increase both in IL-10 and IL-4 [13–15].

Division Followed by Deletion

The demonstration that SAgs encoded by endogenous murine retroviruses caused the deletion of CD4+CD8+ precursors in the thymus provided the first formal evidence for a negative selection of the T cell repertoire based on clonal deletion [16, 17]. It was subsequently shown that the bSAg SEB also caused deletion of CD4+CD8+ precursors in the thymus [18].

Exposure of peripheral T cells either to cells expressing viral SAgs (vSAgs) or to bSAgs causes partial deletion of SAg-reactive CD4+ T cells

[19–21]. Most of the deletion occurs after a phase of T cell proliferation, but some cells may also be acutely deleted [21]. Deletion induced by bSAgs is variable depending on the dose of antigen and the immunization protocol [22, 23]. In general, high doses or repeated low doses of bSAgs induce more massive deletion than a single injection of either bSAg- or vSAg-expressing cells. The deletion of CD8+ T cells is somewhat variable in different experimental models. In some cases, this population is not deleted at all [24], only to a minor extent [7, 19, 20, 25] or is severely deleted [22].

The deletion of SAg-reactive CD4+ T cells is caused by induction of apoptosis [20]. The demonstration that deletion of SAg-reactive peripheral CD4+ T cells is reduced in Fas-deficient animals [26, 27] indicates that the deletion is induced via the Fas/FasL pathway (reviewed in Van Parijs and Abbas [28]). CD8+ T cells have been shown to cause deletion of CD4+ T cells in response to bSAgs [29–31]. In response to SEB, CD8+ T cells specifically killed Vβ8+ SEB-activated CD4+ T cells, but not resting cells [29, 30]. The interaction with CD4+ T cells involved recognition of the nonclassical MHC I molecule Qa-1. CD8+ T cells induced in response to SEA, however, were shown to kill activated CD4+ T cells in a Vβ-unrestricted way. In this case, the killing was Fas/FasL dependent [31].

Anergy

The SAg-reactive CD4+ T cells remaining after the in vivo SAg-induced proliferation and deletion are anergic [32]. In functional terms, 'anergic' means that the T cells do not respond fully to in vitro or in vivo restimulation with the immunizing SAg. Anergy is detected experimentally as reduced T cell proliferation and/or IL-2 production and most often using in vitro settings. In general, the cytotoxic function of CD8+ T cells is not reduced in mice with induced CD4 T cell anergy [12, 33]. Depending on the conditions, the in vitro recall proliferation response of CD8+ T cells may be somewhat reduced, maintaining full cytotoxic activity [12], while using other experimental protocols cytotoxicity is significantly reduced as well [34].

T cell functions are affected to a variable extent in the anergic cells. Some protocols induce only a reduction in T cell division and IL-2 production [32], while others induce profound unresponsiveness even to potent polyclonal T cell activators [35]. Depending on the protocol used for inducing the anergy, CD4+ T cell proliferation can be restored or not by the addition of exogenous IL-2 to in vitro cultures. In vivo exposure to high doses of SAgs [23] or repeated exposure to low doses often cause more profound unresponsiveness to IL-2 [12, 14, 22].

In these cases, the anergic T cells fail to respond to IL-2 even though they may express the high-affinity IL-2R [25, 36, 37].

In vitro versus in vivo Anergy

Most of the initial studies used in vitro proliferation or IL-2 production assays to demonstrate induction of T cell anergy. In some cases, T cell anergy was also shown to operate in vivo [30, 38], but not in others. Thus, in vivo SAg challenge of anergic mice induced secretion of IL-2 and other cytokines in vivo [39, 40]. Further, CD4+ T cells that were anergic when exposed to the antigen in vitro were able to induce efficient B cell responses in vivo [13, 41, 42].

The induction of anergy is an active process since most anergic cells have proliferated in response to the SAg [43]. The molecular basis of SAg-induced CD4+ T cell anergy has been extensively investigated revealing changes in intracellular signaling cascades [37, 44, 45]. Using CD4+ T cells obtained from mice immunized once with SEB, MacDonald and coworkers [46] showed that the SAg-induced anergy was T cell autonomous. However, other reports showed that anergy was not T cell autonomous, but rather depended on other non-T cells suppressing the CD4+ population [35]. Further, the CD4+ population may in some cases be heterogeneous containing both anergic cells and regulatory T cells (Tregs) [36] or Tregs and other CD4+ T cells actively suppressed by the Tregs [47]. Thus, coining a population of CD4+ T cells angergic does not necessarily mean that is a property of all the cells in that population. This point will be further discussed below in the section on SAg-induced Tregs.

Regulatory T Cells

CD4+CD25+ Natural Tregs

The CD4+CD25+ Tregs were first described by Sakaguchi et al. [48]. Their pioneering work demonstrated that these natural Tregs are important in maintaining self-tolerance [48–50]. This cell population has been intensely studied during the past few years and it has been invoked in immunoregulation and tolerance in many different situations both in experimental animals and in man. Thus, these cells have been shown to: (a) inhibit the development of autoimmune diseases, (b) to participate in the establishment of transplantation tolerance, (c) to regulate chronic infections and inflammation, (d) to mediate tolerance to food antigens and inhaled innocuous antigens and (e) to regulate immunity to infection and tumors (reviewed in Sakaguchi et al. [51], Maloy and

Powrie [52], Shevach [53], Cobbold et al. [54] and Mills [55]). Due to the availability of numerous excellent reviews describing the phenotypic and functional characteristics of these cells, only certain aspects that are relevant to the understanding of Tregs in SAg-induced responses will be mentioned in here.

Characteristics of Natural Tregs

The initial functional studies suggested that natural Tregs are anergic in that they respond poorly to antigen challenge in vitro [56, 57]. However, it was subsequently shown that these cells expand well both in vitro in the presence of the appropriate stimuli and in vivo (reviewed in von Boehmer [58]). Since activated 'conventional' T cells also express CD25 (the IL-2 receptor α-chain), other more selective markers for this population were sought for. Natural Tregs express the glucocorticoid-induced TNFR-related gene and CTLA-4 at high levels and both these molecules have been implicated in the function of these cells [59–62]. However, activated conventional CD4+ T cells express both these markers as well. The most selective marker for natural Tregs currently available is the transcription factor Foxp3 [63–65]. Indeed, this protein is essential for the development of natural Tregs and transgenic expression in conventional CD4+CD25− T cells induces their development into natural Tregs. Because of the central role of natural Tregs in maintaining self-tolerance, genetic deficiency in the Foxp3 gene is associated with the development of autoimmune disease both in mouse and in man (reviewed in Fontenot et al. [66] and Sakaguchi [67]).

Development of Natural Tregs

Natural Tregs develop in the thymus upon recognition of self-antigens presented by thymic stroma cells [50, 68–70]. A large number of recent publications have addressed extrathymic development of natural Tregs. Wahl and coworkers [71] initially showed that CD4+CD25− peripheral T cells could be induced to Foxp3-expressing natural Tregs when activated in vitro in the presence of TGF-β_1. Several other laboratories have subsequently confirmed this important observation. One problem of this approach is that CD4+CD25− cells still contain Foxp3-expressing bona fide natural Tregs [72] and it is difficult to judge whether the culture protocol selects for contaminating natural Tregs present from the beginning of culture or whether there is actually de novo induction of these cells.

There is compelling evidence for the extrathymic development of natural Tregs in vivo. In these experiments, investigators have used TCR-transgenic mice crossed to Rag-deficient mice. Depending on the nature of the transgenic TCR, these mice will carry no other lymphocytes than CD4+ T cells and all of these will express the transgene-encoded TCR. Since such animals can be bred

under conditions where the antigen recognized by the transgenic TCR is absent in the thymus (since it is most often a foreign antigen), natural Tregs are absent in these mice [69, 73]. Under experimental conditions in which the mature peripheral TCR-transgenic T cells encounter their antigen presented by immature APCs, some of the CD4+ T cells will differentiate into Foxp3-expressing natural Tregs [74, 75]. This is believed to mimic the situation when naive T cells are exposed to self-antigens after their export from the thymus, since during normal steady-state conditions self-antigens are presented by immature dendritic cells (DCs) (reviewed in Fazekas de St. Groth [76], Roncarolo et al. [77] and Steinman and Nussenzweig [78]).

Functions of Natural Tregs

The functions of natural Tregs have been extensively studied both in vivo and in vitro. The initial in vitro experiments showed that these cells inhibited T cell proliferation in a cell contact-dependent, cytokine-independent way [56, 57]. Conversely, the inhibition of intestinal inflammation by these cells in vivo was reported to be cytokine dependent involving TGF-β_1 and/or IL-10 production depending on the experimental model [79, 80]. Both these cytokines are immunosuppressive and have been invoked in immunologic tolerance (reviewed in Gorelik and Flavell [81] and Moore et al. [82]). A recent report showed that natural Tregs from TGF-β_1-deficient mice also provided protection from intestinal inflammation [83]. However, these Tregs probably induce TGF-β_1 production in other cells since the protection from disease was still TGF-β_1 dependent [83]. Furthermore, it has been proposed that natural Tregs can induce the production of IL-10 in other T cells [84–86]. The in vivo functions of natural Tregs involve inhibition of T cell proliferation [87], T cell effector function [88] and inhibition of inflammatory cells [89] and are all cytokine-mediated effects.

IL-10-Secreting Tregs

Groux et al. [90] showed that human and mouse CD4+ T cells chronically activated in the presence of IL-10 in vitro were anergic. These cells suppressed T cell proliferation in vitro in a cell contact-independent way by producing the immunosuppressive cytokines IL-10 and TGF-β_1. Further, the cells also suppressed the development of experimental colitis in a mouse model [90]. These suppressive cells were denoted T regulatory type 1 cells (Tr1).

Repetitive stimulation with protein antigens [70, 91, 92], with SAgs [14, 15, 36, 93] or with immature allogeneic DCs [94] induces Tr1-like IL-10-secreting Tregs (IL-10-Tregs) in vivo. Another common denominator of these

protocols besides repetition is that the antigens were in all cases presented by immature DCs. This point will be further discussed below. Of particular interest is that IL-10-Tregs can develop in the absence of natural Tregs indicating that these two lineages are developmentally distinct [70, 95]. Further, IL-10-Tregs do not express the Foxp3 gene, providing a marker that distinguishes them from natural Tregs, which may produce IL-10 as well (see above).

While the Tregs induced by repetitive stimulation inhibit T cell proliferation in an IL-10-dependent way in vivo, their in vitro suppression is not always IL-10 dependent [91, 95]. In this respect, they are similar to natural Tregs, which also inhibit T cell proliferation in a cytokine-independent way in vitro [56, 57]. IL-10-Tregs have been shown to inhibit autoimmune disease [96], to regulate allergic responses [97] and responses to infection [98, 99].

In summary, natural Tregs and IL-10-Tregs have common functional features: (a) they both suppress T cell proliferation in vivo and in vitro, (b) they both produce IL-10 and/or TGF-β_1 and inhibit the development of autoimmune disease and (c) they regulate the immune response to infection. Since both natural Tregs and the IL-10-Tregs, depending on the conditions, may or may not express the CD25 marker, these two populations are not always easy to distinguish. Antibodies to the Foxp3 molecule both in mouse and in human provides the best tool currently available to enable such a distinction.

T Helper Type 3 Cells

Oral administration of protein antigens was shown to induce CD4+ T cells secreting TGF-β_1 and variable amounts of IL-10 in mesenteric lymph nodes [100]. These cells were shown to suppress autoimmune disease (reviewed in Weiner [101]). Natural Tregs are also induced in these conditions but T helper type 3 cells may be functionally distinct from this population [102].

Regulatory Cells in Superantigen-Induced Responses

As reviewed above, numerous reports have focused on SAg-induced anergy and deletion of T cells. In contrast, only a few studies have been published that deal with Tregs induced by such antigens. Both CD4+ and CD8+ Tregs have been studied and in addition one laboratory reported that myeloid cells also have regulatory activity in these responses. Since the various regulatory cells have been identified in different experimental models, I will review data from reports with similar experimental outline.

CD4+ Tregs Induced by Repeated Immunization with bSAgs

Several laboratories have reported that mice repeatedly immunized with bSAgs have an increased serum concentration of IL-10, while IL-2, IFN-γ and TNF-α concentrations are reduced [12–15]. Similar data were obtained upon in vitro challenge of CD4+ T cells or spleen cells from such mice with the bSAg [15, 36, 37, 93, 103]. The repeated SAg exposure induces severe deletion of responding CD4+ T cells [60, 93] and anergy [13, 36, 37, 93, 103]. The tolerant CD4+ T cells from SAg-treated animals have a prolonged deficiency in IL-2 production [36]. These cells also respond poorly to IL-2 in vitro, due to initial low expression of the high-affinity IL-2R and defective signaling through that receptor [36, 37]. This explains the anergic phenotype, since CD4+ T cell proliferation in vitro is highly IL-2 dependent (reviewed in Malek [104]).

CD4+ T cells purified from mice made tolerant by repeated exposure to bSAgs have also been shown to inhibit SAg-induced proliferation and IL-2 production of naive responder T cells in vitro [15, 36, 93, 103]. In the reports from Braun et al. [15, 103], SAg-specific tolerance was induced by repeated injection of SEB in normal BALB/c or C57BL/6 mice. The tolerant CD4+ T cells were shown to express high levels of CTLA-4, similarly to natural Tregs [61, 62]. They contained Tregs as they inhibited SEB-induced proliferation and production of IL-2 and IFN-γ by cocultured naive responder spleen cells [15, 103]. In their second report, the authors showed that CD4+CD25− T cells were the effector Tregs, while curiously CD4+CD25+ T cells from the tolerant animals were virtually devoid of regulatory activity [103]. Rather, the CD4+CD25+ population contained IFN-γ-producing effector cells that 'masked' the in vitro regulatory activity of this population. The regulatory activity of the CD4+CD25− T cells correlated with high IL-10 production upon in vitro activation [103]. Notably, natural Tregs from mice exposed to a vSAg have been reported to produce IL-10 [105] and it is known that natural Tregs can loose CD25 expression upon in vivo expansion [87]. However, the CD4+CD25− Tregs studied by Braun and coworkers [103] did not represent such 'modulated' natural Tregs, since they did not express the Foxp3 gene, which is the hallmark of natural Tregs. Thus, these CD4+CD25− Tregs are more similar to the Tr1 cells [90] and peptide-induced IL-10-Tregs [70, 91, 92] as mentioned above.

Importantly, Braun and coworkers [15] showed that spleen cells from the tolerant mice, when transferred to normal recipient mice, inhibited the SEB-induced IL-2 and IFN-γ response in the recipients. This result formally demonstrated that the SEB-specific tolerance in mice exposed repeatedly to SEB is dominant and mediated by suppression. This inhibition could be reversed by in vivo treatment of recipient mice with neutralizing anti-IL-10 antibodies, suggesting that IL-10-secreting cells in the transferred population mediated the suppression. The

authors did not formally show, however, that IL-10-producing CD4+ T cells mediated the suppression, even if that was a likely explanation to their results.

Even though the CD4+CD25+ T cells from tolerant mice appeared devoid of in vitro regulatory activity in the study of Braun and coworkers [103], the presence of natural Tregs in vivo was important, since mice depleted of these cells did not survive the repeated SEB treatment due to induction of toxic shock. This observation made the authors tentatively conclude that tolerance induction by repeated exposure to SEB in vivo requires the presence of CD4+CD25+ T cells (natural Tregs). This point will be further discussed below. Importantly, it was shown that CD4+CD25− T cells purified from tolerant mice could prevent the development of toxic shock in mice depleted of CD25+ T cells. Therefore, the CD4+CD25− T cells in this system are Tregs both in vivo and in vitro and they perform their in vivo function in the absence of CD4+CD25+ T cells.

In our studies, we have used TCR-transgenic mouse models, where the majority of CD4+ T cells express either Vβ3-positive or Vβ8-positive TCRs allowing for the use of SEA or SEB for in vivo tolerance induction [93]. Previous reports had established that three consecutive injections of these SAgs, performed every fourth day, were enough to cause profound unresponsiveness to the immunizing SAg both in vivo and in vitro [12, 14]. In particular, we were intrigued by the high levels of IL-10 secretion observed in these mice, suggesting that the SAg-induced tolerance might be accompanied by a change in T cell effector function. Since it had been suggested that natural Tregs might suppress immune responses in an IL-10-dependent way in vivo [79] and that such cells could be induced by repeated or tolerogenic stimulation with peptide antigens in vivo [102, 106, 107], we thought that Tregs might be part of the anergy phenomenon seen in our model. This idea was further fuelled by the studies of Papiernik et al. [108], who had proposed that natural Tregs are insensitive to activation-induced cell death. We therefore assumed that SAg-reactive T cells that were not deleted during the SAg response would be enriched for natural Tregs. As mentioned above, these cells do not proliferate well when exposed to antigen in vitro, unless a source of IL-2 is present in the cultures. Hence, these cells were at the time regarded as anergic, although that later turned out not to be the case in vivo (reviewed in von Boehmer [58]).

Our data showed that both the CD4+CD25− and CD4+CD25+ T cells from tolerant mice were highly efficient Tregs in vitro [93]. Both populations had similar regulatory functions as natural Tregs isolated from nonimmunized TCR-transgenic or normal mice, but were much more potent on a per-cell basis. Thus, the T cells inhibited T cell proliferation and IL-2 production in vitro. Further, both populations downmodulated CD80 and CD86 expression on DCs, an effector mechanism that we had previously ascribed to natural Tregs [109].

The increased functional efficiency was most likely a consequence of the in vivo preactivation and expansion of these cells, since it has been demonstrated that preactivated natural Tregs are indeed more potent regulatory cells in vitro [110]. Further, because of the functional and phenotypic similarity of these two populations we assumed that the CD25− cells might at least partially represent natural Tregs that had lost CD25 expression. We have subsequently confirmed that both the CD25− and CD25+ populations express the Foxp3 gene [Oderup and Ivars, in preparation], lending further support for this view. However, these populations are likely heterogeneous and the functional activity determined in our assays might be a property of only a fraction of the cells, suppressing the functions of other non-Tregs in the population. We will address this possibility in future experiments using single-cell analyses of Foxp3 expression.

As mentioned above, Braun and coworkers [103] reported that CD4+ CD25− T cells were the dominant regulatory population in SEB-tolerant normal mice. However, both CD25+ and CD25− CD4+ T cells isolated from tolerant thymectomized mice were potent regulatory cells in vitro. The authors therefore suggested that effector CD25+ T cells masked the regulatory function of the CD25+ fraction isolated from tolerant euthymic mice. Since we found that both CD25+ and CD25− CD4+ T cells developed efficient regulatory function in tolerant TCR-transgenic mice [93], the relative content of Tregs may be higher in the CD25+ population in our model. Further experimentation is needed to explain this paradox.

In the report of Miller et al. [36], in vitro SEA-challenged SEA-tolerant CD4+ T cells were shown to produce IL-10 and TGF-β that inhibited the IL-2 production by naive responder T cells. These authors induced tolerance in Rag-deficient TCR-transgenic mice which lack CD4+CD25+ T cells [50, 69, 73] and their CD4+ T cells express the Foxp3 gene at extremely low level [111, 112]. We also used TCR-transgenic Rag-deficient mice in our experiments [93] and obtained data quite similar to those of Miller et al. [36]. Thus, we found that tolerant CD4+ T cells from such mice suppress T cell proliferation largely through the secretion of soluble factors that could be neutralized with antibodies to IL-10 and TGF-β_1. These cells are functionally similar to the Tr1 cells [90] described above and to CD4+ T cells induced by repeated intranasal exposure to peptide antigen [91, 95]. Further, they inefficiently downmodulated the expression of CD80 and CD86 molecules on DCs, distinguishing these cells functionally from CD4+CD25+ cells from normal mice [109] and SEA-tolerized CD4+CD25+ and CD4+CD25− cells from TCR-transgenic Rag+ mice [93]. We have recently found that the SAg-tolerized cells from TCR-transgenic Rag-deficient mice, similarly to the peptide-tolerant T cells, do not express the Foxp3 gene and few cells express the CD25 protein [Oderup and Ivars, in preparation; 95]. Thus, regulatory CD4+ T cells isolated from TCR-transgenic

Rag-deficient mice made tolerant by repeated exposure to bSAgs are distinct from natural CD4+CD25+ Tregs.

Braun and coworkers [103] proposed that CD4+CD25+ T cells may act as inducers of regulatory function in CD4+CD25− T cell SEB-tolerant mice. Nevertheless, the authors also reported that CD4+CD25− Tregs do indeed develop in thymectomized, SEB-tolerant mice depleted of CD25+ T cells albeit in lower numbers. This is fully compatible with our data obtained from the analysis of SEB-tolerant TCR-transgenic Rag-deficient mice, which lack CD4+CD25+ natural Tregs [93]. Thus, CD4+CD25− Tregs can develop in SEB-tolerant animals in the absence of natural Tregs.

CD4+ Tregs Induced by Repeated Exposure to vSAgs

Infectious MMTV transmitted via milk to newborn mice induces both anergy and clonal deletion (reviewed in Acha-Orbea et al. [113]). Papiernik et al. [108] showed that such an infection selectively spared vSAg-reactive CD4+CD25+ T cells. These cells were phenotypically and functionally identical to natural Tregs [105, 108, 114]. The Tregs were shown to reduce the cytokine burst induced by bSAg in an IL-10-dependent way [105] and were proposed to protect T cells from vSAg-induced clonal deletion.

Greene and coworkers [47] have characterized Tregs induced in Vβ8.1 TCR-transgenic mice injected two consecutive times with Mls-1^a-positive B cells. These Tregs were also shown to be CD4+CD25+ cells that suppressed the Mls-1^a-induced proliferation and IL-2 production by naive transgenic CD4+ T cells. The in vitro suppression was cell contact dependent and could be reversed with the addition of exogenous IL-2. These data are consistent with the view that these cells represent bona fide natural Tregs. Interestingly, the natural Tregs were demonstrated to suppress a residual nonanergic population of Mls-1^a-reactive CD4+ T cells present in the tolerant cell population. Natural Tregs thus actively maintained the tolerance of these latter antigen-reactive cells (fig. 2).

Other SAg-Induced Regulatory Cells

As mentioned above, CD8+ T cells induced in SAg responses may kill activated CD4+ T cells and therefore perform a regulatory function in the response. Further, Swain and coworkers [35] have reported that CD4−CD8− cells suppressed CD4+ T cells in an IFN-γ-dependent way. A subsequent study from the same laboratory provided evidence that myeloid cells mediated that regulatory mechanism [115].

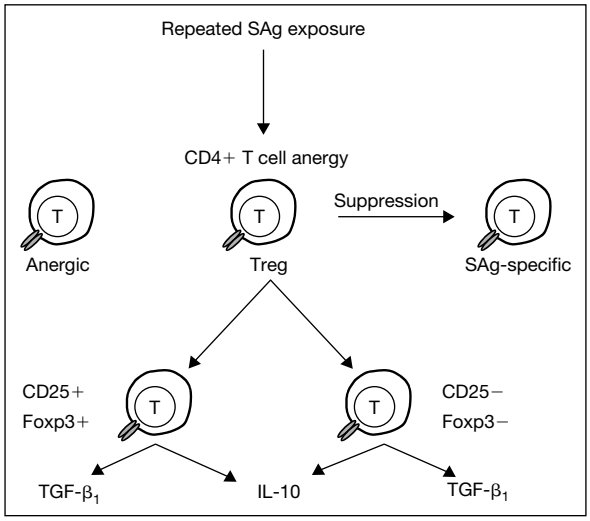

Fig. 2. Heterogeneity of the anergic CD4+ T cell population in mice repeatedly exposed to SAgs. The anergic cell population contains both inherently SAg-unresponsive (anergic) cells, Tregs and SAg-specific cells actively suppressed by the Tregs. The Treg population may contain both natural CD4+CD25+ Foxp3+ Tregs and CD4+CD25− Foxp3− IL-10-Tregs. The composition of the anergic population may vary in different experimental models.

Collectively, the available data suggest that SAg-induced tolerance is maintained both by anergy and suppression of reactive cells (fig. 2). The level of anergy and suppression may vary in the various experimental models used and might explain why tolerance is sometimes seen together with other effector functions such as B cell activation. Further, the relative proportions of natural Tregs versus IL-10-Tregs may vary depending on the protocol used for tolerance induction.

What Cells Present SAgs in vivo?

SAgs do not need to be processed in order to induce T cell activation, while MHC II expression is important for their presentation. In vivo the most common MHC-II-positive cell is the B cell. Indeed, the endogenous vSAg Mls-1[a] (Mtv-7) is primarily presented by B cells [116] and vSAg-induced deletion of peripheral T cells is impaired in B-cell-deficient animals [117]. However, Vacheron et al. [118] showed that also DCs present vSAgs from an infectious type retrovirus. The observation that precursor T cells reactive to endogenous

retroviruses are deleted during development in the thymus of B-cell-deficient animals further supported that these vSAgs can be presented on other cells than B cells [119]. Most importantly, DCs present SEA and induce T cell activation much more efficiently than B cells and monocytes [120, 121]. Consequently, even though intravenous injection of bSAgs would target MHC-II-positive cells systemically, the bSAgs might be most efficiently presented to naive T cells by the resident DCs in lymphoid organs. Consistent with this, SEB induces T cell activation and tolerance also in B-cell-deficient mice [122].

Naive DCs and B Cells Induce Tolerance

B cells express low levels of the CD80 and CD86 costimulatory molecules, which are important for the activation of naive T cells. Indeed, resting B cells do not adequately activate naive T cells and induce tolerance rather than immunity [123, 124].

The majority of resident DCs are immature cells expressing low levels of costimulatory molecules. Such DCs induce T cell proliferation, but do not cause efficient maturation of T cell effector functions [125, 126]. The consequence of SEB exposure on splenic APCs has been described [127, 128]. Thus, SEB caused a rapid redistribution of DCs from the marginal zone to the periarterial lymphatic sheath where T cells reside. Further, the DCs upregulated expression of the CD80, CD86 and CD40 costimulatory molecules. No upregulation was seen on B cells and monocytes, supporting the view that DCs might indeed be the major presenter of the bSAgs [127, 128]. Muraille et al. [128] showed that the expression level of CD80 and CD86 was considerably lower on SAg-activated DCs than on lipopolysaccharide-activated DCs [127, 128] and was both T cell and MHC II dependent. It is therefore likely that the bSAgs are presented by DCs that provide less costimulation than DCs activated by microbial products such as lipopolysaccharide.

Similar in vivo T Cell Responses to SAgs and Conventional Protein Antigens

Immunologists have for several decades used globular proteins as model antigens to study experimental immunological tolerance in vivo. To induce tolerance, the proteins can be administered orally (‘oral tolerance’) or by injection either intravenously or intraperitoneally. The protein must be delivered in the absence of adjuvants, which would otherwise activate the antigen-presenting

DCs, and cause the generation of an immune response resulting in the formation of both effector and memory T cells.

In vivo T cell responses to protein antigens or synthetic peptides thereof, in the absence of adjuvants, are remarkably similar to the immune responses to SAgs (fig. 1). The use of homogenous CD4+ T cells from TCR-transgenic mice has allowed researchers to study primary responses to peptide antigens in vivo at a high level of resolution. Several laboratories have shown that such monoclonal peptide-specific T cells proliferate in response to the peptide, accumulate and subsequently decrease in number [102, 114, 125, 129]. The peptide-specific T cells remaining after the contraction phase are anergic when tested for their in vitro responsiveness to the peptide [74, 91, 102, 114, 125]. Similarly, CD4+ T cells also divide in response to transgene-encoded 'self-antigens' and such T cells also become anergic to further exposure to those antigens [70, 130–132]. Such anergic peptide-specific T cells inhibit the peptide-specific proliferation response of naive T cells in recipient mice. Hence, this population contains Tregs, which in a dominant fashion can impose the state of tolerance upon naive peptide-specific T cells.

Under these conditions, the self-antigen is presented by APCs that have not been deliberately stimulated by inflammatory signals. Thus, the APCs provide deficient costimulation to the responding T cells, and induce a tolerogenic T cell response (reviewed in Fazekas de St. Groth [76], Roncarolo et al. [77] and Steinman and Nussenzweig [78]). Interestingly, the simultaneous immunization of animals with peptide antigen in adjuvant and exposure to SEA prevented the deletion of Vβ3+ CD4+ T cells [133]. Exposure to adjuvant will activate peptide-presenting DCs locally at the site of immunization and these cells will in turn activate peptide-specific T cells in a local draining lymph node (reviewed in Akira et al. [134]). These T cells will thus be activated by DCs that are functionally distinct, providing a possible explanation as to why T cells escape deletion in the lipopolysaccharide-modulated SAg response [135]. Consistent with the above explanation is also the observation that SEB does not induce tolerance in animals injected with IL-1 [136], which activates DCs and can functionally replace adjuvants in vivo.

In summary, the in vivo T cell response to SAgs and to conventional protein antigens is very similar, when the protein antigen is presented by immature DCs. Both agents cause initial T cell proliferation, followed by clonal deletion and anergy of remaining reactive T cells. The anergic T cell population in both cases includes antigen-specific Tregs that can suppress the response of naive antigen-specific T cells both in vivo and in vitro. Further, the population includes anergic cells that are inherently antigen nonresponsive. SAgs activate a much larger fraction of the T cell repertoire in a normal animal than a peptide antigen and the phenomena of anergy and deletion were therefore initially much

easier to visualize and study using SAgs. This difference was overcome by using TCR-transgenic T cell populations in which virtually all CD4+ (or CD8+) T cells would respond to the peptide antigen.

What Might Be the Role of Tregs in SAg-Induced Responses?

Tregs induced in SAg responses produce IL-10 and TGF-β_1 [15, 36, 93, 103], which are both immunosuppressive cytokines (reviewed in Gorelik and Flavell [81] and Moore et al. [82]). IL-10 plays a crucial role in the SEB response by dampening the inflammatory response and protecting immunized animals from shock [137]. The SEB-induced IL-10-Tregs were indeed shown to protect SEB-immunized animals from toxic shock syndrome [103]. Importantly, IL-10 treatment has also been shown to induce tolerogenic, IL-10-producing DCs [138]. Such DCs induce Tr1-like IL-10-Tregs [138] and antigen-specific tolerance in vivo [138, 139]. During normal steady state, splenic stromal cells induce the differentiation of such DCs [139]. As already mentioned above, Cauley et al. [115] reported that a myeloid cell population derived from SAg-immunized mice has a regulatory capacity, with the ability to inhibit SAg-specific responses of naive T cells. Further, DC maturation in the SEB response is T cell dependent [128] indicating that T cells and DCs actively communicate in the response. One might speculate that the CD4+CD25− IL-10-Treg populations detected in SAg-immunized mice [36, 93, 103] may be induced by such tolerogenic DCs. Thus, IL-10 might be involved both in regulating the SAg-induced immune response and in the generation of IL-10-Tregs during the response. The possible roles of TGF-β_1 are less clear but it could be that Treg-produced TGF-β_1 may induce conventional CD4+CD25− T cells to acquire the function of Tregs [71, 83] or to play a general immunosuppressive role. The CD4+CD25+ Tregs downmodulate costimulatory CD80 and CD86 molecules on DCs in vitro [93], but is not known whether this is also an effector function of these cells in vivo. Natural Tregs have been shown to regulate T cell expansion [87] and it is possible that this population may play a similar role in the immune response to SAgs although that possibility has not been experimentally addressed to my knowledge.

Concluding Remarks

SAgs induce strong in vivo immune responses involving massive production of inflammatory cytokines. Unless regulated by Tregs producing immunosuppressive cytokines the response may cause death. One interesting

question that remains to be answered is why SAgs sometimes induce natural Tregs while in other cases CD4+CD25− IL-10-Treg dominate. Thus, repeated exposure to SAgs appears most efficient in inducing IL-10 production and may mimic chronic infection by the SAg-expressing microbe. Conversely, in animals carrying infectious MMTV natural Tregs appear to be dominant. The development of IL-10-Tregs does not require the presence of natural Tregs. Is the induction of these cells mediated by tolerogenic DCs and if so what is the relationship of these to other tolerogenic DC populations? Further, what is the role of repeated SAg exposure in Treg induction? DCs inducing IL-10-Tregs have been described and it would be interesting to address whether such DCs also operate in the generation of the SAg-induced IL-10 response. These and other remaining questions will hopefully inspire to further experimentation in this field.

Finally, I would like to apologize to the authors of all relevant papers that I could not refer to because of the space limitations of this article.

Acknowledgements

Our work was supported by grants from the Swedish Research Council grant number K2005-06X-14184-04A, Crafoordska Stiftelsen, Alfred Österlunds Stiftelse, Greta och Johan Kocks Stiftelse. I would like to thank Dr. Anette Sundstedt for helpful comments on the manuscript.

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Marone G (ed): Superantigens and Superallergens.
Chem Immunol Allergy. Basel, Karger, 2007, vol 93, pp 161–180

T Cell Signalling Induced by Bacterial Superantigens

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Abstract

Bacterial superantigens (SAgs) constitute a large family of bacterial toxins that share the capacity to induce massive activation of the human immune system. Such a feature is based on the ability of these toxins to activate T cells that express β-chains of the T cell antigen receptor (TCR) containing variable regions (V) coded by specific families of Vβ genes. In addition, bacterial SAgs bypass the need for processing by antigen-presenting cells by directly binding to major histocompatibility complex class II molecules on the surface of these cells. Emerging work indicates that bacterial SAgs utilize not only the canonical pathways of TCR-mediated T cell activation but also other pathways. Here, we review the structural information on recognition of bacterial SAgs by T cells, the TCR signalling induced by this recognition event, and the effector functions that this recognition triggers. We analyze experimental evidence suggesting the existence of alternative receptors and coreceptors for bacterial SAgs, and outline future challenges in the research with these toxins.

The term ‘superantigen’ (SAg) was first introduced in 1989 to describe the ability of the staphylococcal enterotoxin B (SEB) to induce a remarkable expansion of T cells expressing T cell receptors (TCRs) with a specific subset of TCR β-chain variable (V) regions [1]. Such a response was qualitatively and quantitatively distinct from the T cell response to conventional antigens. Typical antigens are processed inside antigen-presenting cells (APCs) into small peptides of 8–20 amino acids that bind a membrane-distal groove in the molecules of the major histocompatibility complex (MHC) [2]. The peptide/MHC complexes are then exported to the APC surface, where they act as ligands of TCR

on a few specific T cell clones. The recognition of peptide/MHC complexes is mediated by both TCR α and TCR β variable domains (Vα, Jα, Vβ, Dβ, Jβ). In contrast, most bacterial SAgs bind directly to the lateral surfaces of the MHC class II molecules and to the Vβ domain of the TCR and thus bypass the requirements for processing and presentation of conventional antigens [3–6]. The net result of these differences in recognition is that conventional antigens may stimulate in the order of less than 1 in 10,000–100,000 T cells while SAgs can stimulate up to 20% of all T cells. Therefore, the frequency of T cells responding to SAg surpasses that of conventional peptide antigens by multiple orders of magnitude. In addition, these bacterial toxins are extremely potent in that significant T cell proliferation is detected for some SAgs in the picogram (10^{-12} g)/milliliter range. The immediate outcome of the T cell recognition of bacterial SAgs is the large expansion of T cells with the resulting massive release of cytokines by the host, which are believed to be responsible for the most severe consequences of SAg-associated diseases [7]. Furthermore, following the initial expansion, the host may become unresponsive to the SAg by several mechanisms including T cell anergy and activation-induced clonal T cell deletion [1].

Staphylococcal SAgs belong to the large class of pyrogenic toxin SAgs that are also produced by *Streptococcus pyogenes* and some other β-hemolytic streptococci, and together these represent the best-characterized class of SAgs [7]. There are now in excess of 30 distinct serotypes of staphylococcal and streptococcal SAgs [8]. These toxins are ribosomally synthesized proteins of relatively similar molecular weight (approx. 22–28 kDa). Although staphylococcal and streptococcal SAgs generally share only a low percentage of sequence homology, they all seem to share a similar three-dimensional fold based on crystallographic studies [9]. Each characterized structure contains an N-terminal β-barrel oligosaccharide/oligonucleotide-binding fold domain, and a C-terminal β-sheet structure known as the β-grasp domain, with the two domains being joined by a central α-helix [9]. An important aspect to understand some of the biological effects of SAgs is that they can cross-link MHC class II molecules and TCRs by binding to surfaces that are unconventional for typical peptide recognition. It is important to note that the different SAgs interact with these cell surface molecules in several different ways despite the overall conserved structure of the SAgs. In this review, we will go over the structural, biochemical and functional responses of T cells upon recognition of bacterial SAgs. Most of the experimental evidence in this review will refer to pyrogenic toxin SAgs from staphylococci and streptococci. It is not known whether the same or similar patterns of signalling and response are applicable to other SAgs, including mycoplasma SAgs and those of viral origin, since their structural features are different [10, 11].

Structural Biology of Superantigen Recognition by T Lymphocytes

T cell recognition of bacterial SAgs is unique in the sense that it results from interaction of a single chain of the TCR with the SAg interacting also with the MHC molecule outside the peptide-binding groove. As such, T cell activation by SAgs involves the formation of a trimolecular structure primarily dependent on the SAg molecule interacting with the MHC molecule and the TCR molecule. Therefore, we will review the structural information on each of these two interactions, as well as the overall architecture that results from these interactions.

SAg Interaction with MHC Class II Molecules

SAgs bind directly to MHC class II molecules without requiring processing by the APCs. Bacterial SAgs possess two independent binding interfaces, commonly referred to as the low- and high-affinity binding interfaces. The binding affinities for each of them are K_d values of $\sim 10^{-5}$ M for the low-affinity binding interface, and $\sim 10^{-7}$ M for the high-affinity binding interface [9]. The low-affinity binding interface has been characterized crystallographically for the interaction of HLA-DR1 with SEB [12], with the toxic shock syndrome toxin-1 (TSST-1) [13], with the staphylococcal enterotoxin A (SEA) [14] and with the staphylococcal enterotoxin C_3 (SEC_3) [15]. All the low-affinity interactions occur through the oligosaccharide/oligonucleotide-binding fold domain of the SAg to the lateral surface of the domain 1 of MHC class II α-chain. Of all these complexes, the ones involving SEA, SEB and SEC_3 bound similarly. TSST-1, however, had an altered orientation of binding to HLA-DR1 where it bound further up on the MHC class II α-chain, made multiple contacts with the antigenic peptide, as well as with part of domain 1 of the MHC class II β-chain.

In contrast to the low-affinity interaction site of SAg with the MHC class II molecule, the high-affinity binding interface has been characterized crystallographically for fewer SAgs, and currently is only available for the interaction between HLA-DR2a and streptococcal pyrogenic exotoxin C (SpeC) [16], and between HLR-DR1 and staphylococcal enterotoxin H (SEH) [17]. The architectures of these two high-affinity binding modes were very similar and involved contacts between the β-grasp domain of the SAg and domain 1 of the polymorphic MHC class II β-chain, and the N-terminus of the antigenic peptide. Each interaction was coordinated by a zinc ion with three SAg residues and His81 from MHC class II. Approximately one third of the buried surface was with the bound peptide, which is comparable to the contribution of the peptide in typical MHC-TCR interaction.

Multiple studies indicate that T cell activation by some, but not all SAgs, can be influenced by the antigenic peptide, and this may provide a mechanism

for high-affinity binding, but at low density [18]. Otherwise, high-affinity, high-density binding may induce T cell apoptosis [19]. Alternatively, this outcome may be beneficial for the infecting microbe as an immune evasion strategy. Although T cell recognition of bacterial SAgs is not MHC restricted in a classical sense, isotypic and allelic differences in MHC class II molecules influence SAg presentation. Moreover, it has been demonstrated that class II-associated peptides could play a role in SAg presentation. TSST-1 has been shown to induce higher levels of IL-2 when presented by APC pulsed with $SEB_{121–136}$, whereas APC pulsed with $HN_{418–433}$ enhanced IL-2 production in response to SEA but it did not show any effect in TSST-1 responses [20].

SAg Interaction with the TCR β-Chain

The hallmark of the SAg is the ability to stimulate T cells in a Vβ-specific manner such that individual SAgs will activate only certain subsets of T cells, and generally these activation 'fingerprints' are different for dissimilar SAg serotypes. Cocrystallization structures of SAgs in complex with TCR β-chains have been determined for SEC_3 [21], SEB [4], SpeA [6] and SpeC [6]. Although highly homologous SAgs, such as SEB, SEC_3 and SpeA, may have similar architectures for binding to the murine Vβ8.2 chain, SpeC in complex with human Vβ2.1 involved a significantly larger contact surface, a different orientation, and contacts with the CDR3 loop, whereas SEB, SEC or SpeA had no intermolecular contacts with this loop [6]. It also appears that there are significant differences in the binding interfaces and architectures for less similar SAgs, such as TSST-1 [22], and it is likely that as other interactions are characterized, further variation will be seen.

SAg-Mediated T Cell Activation Complexes

The first TCR-SAg cocrystal structure was reported with SEC_3 [21], and by superimposition of the SEB-MHC [12] and SEC_3-TCR [21] complexes, the first complete model of how bacterial SAgs bypass the normal antigen presentation process was revealed [21]. This model predicted that the SAg displaces the TCR and MHC class II molecules like a 'wedge', such that there are no direct contacts between the TCR and the antigenic peptide. In this model, unconventional TCR-MHC contacts did however occur between the TCR α-chain and the MHC class II β-chain that were later shown to provide important stabilizing forces to the trimeric complex [23]. Those SAgs that only possess a low-affinity MHC class II binding domain (e.g. SEB, SEC_3, TSST-1) are each believed to form the ternary 'wedge' complex allowing for the avoidance of peptide specificity, and through the interaction with the TCR β-chain resulting in Vβ-specific T cell activation (fig. 1). The SEB/TCR complex revealed a similar binding mode to SEC_3 where all hydrogen bonds involved main chain

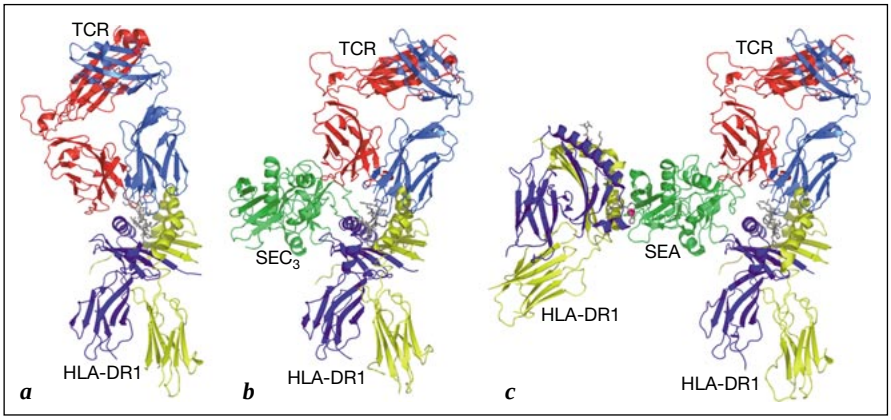

Fig. 1. Ribbon diagrams of typical TCR recognition of a peptide/MHC class II and models of SAg-mediated T cell activation complexes. ***a*** Typical peptide recognition by an α/β TCR where all variable regions of the TCR (Vα, Jα, Vβ, Dβ, Jβ) recognize the antigenic peptide [78]. ***b*** The 'wedge' ternary model where SEC_3 (shown in green) displaces the TCR β-chain away from the MHC class II α-chain and prevents direct contacts of the CDR loops with the antigenic peptide. The model was created by superimposing both the SEC_3-mouse Vβ8.2 cocrystal with the SEC_3-HLA-DR1 cocrystal [15, 21]. Note the unconventional contacts that occur between the TCR α-chain and the MHC class II β-chain that are required to stabilize the complex [23]. ***c*** A quaternary model where SEA (shown in green) forms a wedge similar to SEC_3, but also cross-links a second MHC class II molecule through a high-affinity, zinc-dependent interaction (zinc ion shown in pink) with the polymorphic MHC class II β-chain. The model was created by superimposing the SEA-MHC class II (low-affinity) cocrystal with the SEH-MHC class II (high-affinity) cocrystal to orientate the high-affinity MHC class II [14, 17] and uses the SEC_3-TCR cocrystal to orientate the TCR. Colors are as follows: TCR α- and β-chains are shown in light blue and red, respectively; MHC class II α- and β-chains are shown in dark blue and yellow, respectively; antigenic peptides are shown in gray; SAgs are shown in green and the zinc ion is shown in pink. The figures were created using Pymol (www.pymol.org).

atoms of mVβ8.2 and thus binding may have been based on conformation and not on individual residue side chains [4]. The structure of SpeA in complex with mVβ8.2 was also similar to the SEB-SEC_3 complexes, except that SpeA also contacted CDR1 and many of the hydrogen bonds occurred with side chain atoms of mVβ8.2 [6]. There are also other indications that the wedge model does not appear to have a single uniform architecture, since TSST-1 interacts differently with MHC class II molecules compared with SEB/SEC_3. Furthermore, TSST-1 also has an altered TCR binding interface as partially defined by mutational analysis, yet is still hypothesized to form a wedge between the two immune receptors [22].

Collectively based on the cocrystallization studies, other models have been proposed to explain how different SAgs can cross-link TCR with MHC class II [4, 6, 14–16]. Although complete cocrystal structures do not exist for these models, they are likely accurate since SAgs do not undergo major structural alterations upon interaction with either ligand [4, 6, 12–14, 16, 17, 21]. Of those SAgs that contain the high-affinity MHC class II binding interface, many may form a quaternary complex capable of cross-linking two MHC class II molecules on the surface of the APC, as well as the TCR (e.g. SEA, SEE; fig. 1). Some SAgs which contain the high-affinity zinc-binding motif, such as SpeC and SEH, have been proposed to lack the low-affinity MHC class II binding interface and may essentially form a 'bridge', without any direct contacts between TCR and MHC class II [14]. However, the presence [24–26] or absence [27] of a low-affinity MHC class II binding domain in SpeC remains controversial, but may be related to the specificity of some low-affinity MHC class II binding interfaces for HLA-DQ over HLA-DR [28]. Alternatively, SED has been proposed to dimerize through the high-affinity MHC class II binding domain [29, 30], and may interact exclusively through the low-affinity site to cross-link MHC class II, potentially forming a different SAg-mediated T cell activation complex. However, regardless of the architecture of these different activation complexes, the specific orientation of the TCR and MHC class II molecule interactions may be less important for signalling events, but more crucial for the specificity of the activated T cell. On the other hand, those SAgs with the ability to induce high-order oligomerization of MHC class II molecules and, therefore, of the TCR, may potentially have unique signalling/activating properties [31, 32].

Current knowledge of TCR-SAg-MHC class II molecule interactions has led to the proposal that SAgs can be divided into three groups based on their selectivity for TCRs [6]. The first group is highly promiscuous and recognizes TCR β-chains in a conformation-dependent recognition of the CDR2 loops, and no interactions occur with CDR1 or CDR3 (e.g. SEB, SEC_3). The second group is more specific and uses both recognition of conformation and specific side chains, and interacts with both CDR1 and CDR2 (e.g. SpeA). The last group is highly specific and recognizes β-chains based on noncanonical CDR loops and utilizes all three CDR loops (e.g. SpeC).

A clear evolutionary role for SAgs in the persistence and survival of the bacterial pathogen remains elusive, yet since these toxins target both TCR and MHC class II molecules, it is clear that they must be involved in the subversion of the immune system. Relatively low affinities (K_d values of approx. $10^{-4}-10^{-6}$ M) and fast association and dissociation kinetics ($>10^4$ M^{-1} s^{-1} and $>10^2$ s^{-1}, respectively) of SAgs for the TCR β-chains [33] seem to mimic those of conventional TCR-MHC class II peptide interactions. It seems as though

these toxins have evolved to activate as many T cells as possible with extreme potency and variable architecture. The clear involvement of these proteins in severe disease, such as toxic shock syndrome, is likely an overt and aberrant result of their excessive production during severe infection, while their true evolutionary role remains unknown.

T-Cell-Receptor-Dependent Signalling in Response to Bacterial Superantigens

It has been assumed that TCR-dependent signalling by bacterial SAgs follows the same paradigm applicable for the activation of T cells by nominal antigen (reviewed in Samelson [34] and Huang and Wange [35]; fig. 2). Briefly, this paradigm is based on the activation of the src family protein tyrosine kinase Lck that, in most mature peripheral blood T cells, is associated with the CD4/CD8 coreceptor molecules. The mechanism(s) leading to the activation of Lck is (are) only partially known, and involve(s) the dephosphorylation of a negative regulatory tyrosine residue (Y505) by CD45, and its recruitment by CD4/CD8 to the engaged TCR/CD3 complex by virtue of the binding of these coreceptors to MHC molecules presenting the antigenic peptide. The precise molecular architecture that supports Lck activation leading to TCR signalling likely involves the initial formation of dimers of TCR complexes, one engaged with an agonist peptide/MHC complex and the other with an endogenous peptide/MHC complex, bridged by a coreceptor molecule [36]. Once Lck is activated, it phosphorylates the immune receptor tyrosine-based activation motifs on the TCR-associated CD3 chains, which then become sites for the recruitment and activation of the Syk family tyrosine kinase known as ZAP-70. Activated ZAP-70 then phosphorylates multiple tyrosine residues on the cytoplasmic portion of the transmembrane adapter linker of activated T cells (LAT) that act as docking sites for the assembly of multimolecular signalling complexes or signalosomes that activate downstream signalling pathways leading to the activation/nuclear translocation of transcription factors (reviewed in Samelson [34] and Huang and Wange [35]). This series of events occurs in a temporally and spatially regulated interface between the T cell and the APC, known as immunological synapse, and correlates with the formation of cell membrane microdomains determined by protein-protein interactions [37] and/or by cytoskeleton-dependent clustering of lipid rafts [38, 39].

SAgs trigger a similar cascade of events from the TCR as those resulting from signalling induced by nominal antigen, as evidenced by studies using bacterial SAgs as potent T cell activators in multiple experimental systems, particularly those utilizing well-characterized T cell lines (e.g. Jurkat T cells). With

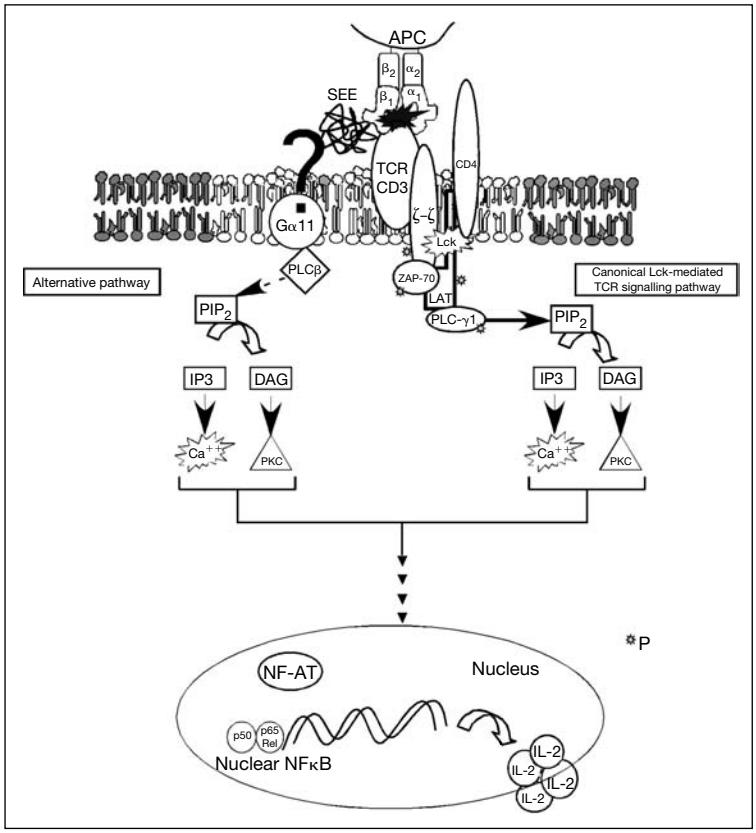

Fig. 2. Two signalling pathways from the TCR are triggered by bacterial SAgs. The canonical Lck-mediated signalling pathway, also used by conventional peptide/MHC complexes, involves tyrosine phosphorylation events following TCR engagement, and leads to the activation of PLCγ-1 and the subsequent increase in intracellular Ca^{2+} and PKC activation. The alternative pathway involves a $G\alpha_{11}$-dependent PLCβ activation leading to increases in intracellular calcium and PKC activation. Both the alternative $G\alpha_{11}$-dependent signalling pathway and the canonical pathway lead to the activation and translocation of transcription factors that ultimately determine changes in gene expression that characterize activated T cells. It is still unclear how bacterial SAgs are able to activate $G\alpha_{11}$, as well as the potential involvement of a coreceptor molecule different from CD4/CD8.

these systems, many groups have shown that SAgs induce all the changes associated with conventional activating TCR signalling, including the formation of putative mature immunological synapses, and the development of effector T cells [40–42]. Thus, early upstream signalling induced by SAgs involves very similar events to those following signalling induced by nominal antigen [42].

However, there is evidence suggesting that TCR signalling in response to SAgs entails some additional unique features. Most of these bacterial toxins are recognized in the context of MHC class II molecules. Therefore, and by analogy with T cell recognition of conventional antigens, one would expect a contribution of the CD4 coreceptor molecules to T cell activation by these toxins because the binding of CD4 to MHC class II molecules stabilizes TCR-mediated recognition and initiates TCR-dependent signalling. Functionally, this would translate into CD4+ T cells constituting the main bulk of the responding T cells to bacterial SAgs. However, there is a body of evidence accumulated over the past years indicating that CD8+ T cells respond as well as CD4+ T cells to some SAgs [43–45]. This includes in vitro data using proliferation, cytokine production and cytotoxic T cell responses as readout for staphylococcal enterotoxin-induced activation of CD4+ T cells and CD8+ T cells, as well as in vivo data showing that injection of SEB into mice leads to a dramatic increase in Vβ8-expressing CD4+ T cells and CD8+ T cells in the spleen, lymph nodes and peripheral blood [43–45]. Together this suggests that the conventional role of CD4 as coreceptor is not operational for the recognition of bacterial SAgs.

Further indication that the CD4 coreceptor is not required for human T cell responses to bacterial SAgs is illustrated by the observation that the responses to at least some staphylococcal SAgs (e.g. SEB, SEC_1) are not affected by blockade of CD4 with monoclonal antibodies against this coreceptor [46]. In our hands, the list of staphylococcal and streptococcal SAgs that are CD4 independent includes, in addition to SEB and SEC_1, SEA, SEE, SME-Z, SpeC, SpeI, SpeJ and TSST-1 [Bueno et al., unpubl. data]. In addition, T cell lines lacking expression of CD4 do respond to bacterial SAgs to a similar magnitude as CD4-expressing T cell counterparts [46, 47]. The lack of CD4 dependency may reflect a true coreceptor independence or, alternatively, just reflect the existence of (an)other molecule(s) that play(s) the coreceptor role instead of CD4. Either way, these findings emphasize the unique character of TCR-mediated recognition of bacterial SAgs and clearly document the need to examine how these bacterial toxins trigger TCR signalling.

Since one of the functions of the CD4 coreceptor molecule is to recruit and facilitate the activation of Lck to initiate signalling from the TCR, it is reasonable to propose that the CD4 independence of T cell responses to bacterial SAgs reflects an absence of Lck requirement to initiate TCR signalling. This hypothesis has been corroborated with extensive data showing that bacterial SAgs such as SEE can activate primary T cells and T cell lines without functional Lck [41, 42, 48]. Such data have been generated with strategies as diverse as pharmacological inhibition of Lck activity, genetic Lck deficiency, manipulation of Lck expression with repressible Lck transfection systems, and small RNA interference for Lck. Under these circumstances, we have reported that not only is

Lck dispensable for the initiation of T cell responses to bacterial SAgs but it actively downregulates SAg-induced T cell activation by triggering a negative regulatory feedback on TCR signalling [41].

It has been suggested that bacterial SAgs could utilize Fyn, the other Src kinase expressed in mature T cells, to initiate signal transduction from the TCR [42]. The use of Fyn could explain the lack of coreceptor dependency of SAg responses since Fyn is not associated with CD4 or CD8 at high stoichiometry but is mostly associated with the TCR complex [49, 50]. However, the alternative use of Fyn to explain the ability of bacterial SAgs to activate Lck-deficient T cells is unlikely since conventional antigens fail to bypass Lck and activate Lck-deficient T cells as one would expect if Fyn were active. Furthermore, primary human T cells respond to bacterial SAgs by producing significant levels of IL-2 in the presence of a selective Src kinase inhibitor PP2, which inhibits both Lck and Fyn [41].

The ability of SAgs to activate Lck-deficient T cells raises the question about an additional alternative signalling pathway(s) emanating from the TCR that is (are) utilized by these bacterial toxins under normal conditions. Determining which SAg-triggered signalling events are not Lck dependent should help us characterize such a pathway. So far, we have shown that membrane-associated events such as T cell-APC conjugation, immunological synapse formation, TCR internalization, and upregulation of CD69 expression in response to bacterial SAgs are comparable between Lck-expressing and Lck-deficient T cells, implying that these events do not require the activity of Lck [40, 41]. More importantly, tyrosine phosphorylation signalling events dependent on Lck activation, which are required for T cell activation by nominal antigen, are dispensable for T cell activation by SAgs. In other words, SAgs can still induce T cell responses in the absence of ZAP-70 activation, LAT phosphorylation and phospholipase (PL) C-γ1 activation [41; unpubl. data]. It is important to keep in mind that, if Lck is available, SAgs do trigger these early signalling events; however, they are not required for SAg-induced T cell responses.

The alternative TCR-dependent signalling cascade used by SAgs converges with the canonical Lck-dependent pathway at the level of ERK-1/2 activation, as indicated by the observation that blockade of activation of ERK-1/2 inhibits T cell responses to these toxins (fig. 2). Downstream, one finds activation and translocation of NF-AT and NF-κB, both in Lck-deficient and in Lck-expressing T cells. Of interest, the contribution of the canonical pathway to this alternative pathway at the level of the activation of ERK-1/2 seems to be primarily quantitative because the magnitude of ERK-1/2 activation in the absence of Lck is lower but the temporal profile of activation is comparable [41].

How can TCR engagement with SAgs activate ERKs in the absence of Lck-dependent early tyrosine phosphorylation/activation events? Based on evidence

presented by Yamasaki et al. [42] and our own, the alternative signalling pathway induces an increase in intracellular Ca^{2+}. In addition, we have observed that inhibitors of protein kinase C (PKC) block the response to bacterial SAgs while inhibitors of phosphatydylinositol 3-kinase have no effect on this response. Ca^{2+} influx and PKC activation are usually linked to a PL. In the canonical pathway of TCR signalling by peptide/MHC, this role is played by PLC-γ1. However, this enzyme is not activated in the absence of Lck activity. Thus, another PLC is involved in the response to bacterial SAgs. Preliminary data indicate that such a role is played by PLC-β [Bueno and Madrenas, unpubl. obs.] (fig. 2). Indeed, we have observed that pharmacological inhibition of PLC-β almost completely prevents ERK-1/2 activation in Lck-deficient T cells in response to SEE. The involvement of PLC-β in the T cell response to bacterial SAgs raises the possibility that the early signalling events include, in addition to Lck activation, the activation of a G protein of the $G\alpha_{q/11}$ family since PLC-β is regulated by heterotrimeric G proteins of this family [51]. Of interest, $G\alpha_{11}$ proteins have already been implicated in TCR signalling under conditions of high degree of cross-linking of the TCR complex [52, 53]. Current work is under way to characterize this G protein-dependent pathway in depth, and identify the mechanisms by which bacterial SAgs such as SEE can activate G proteins (see note added in proof).

Costimulation and late downstream events leading to effector T cell responses are two other aspects to consider when discussing T cell activation. The need for CD28-dependent costimulation to induce T cell proliferation by bacterial SAgs is minor compared to that seen for conventional antigens. However, CD28 costimulation synergizes with TCR signalling by SAgs to increase the potency of the response [54, 55], suggesting that the alternative TCR signalling pathway(s) utilized by bacterial SAgs is (are) distinct from the one used by CD28. The late biochemical responses to bacterial SAgs in T cells, such as upregulation of activation markers, adhesion molecules, induction of the expression of the IL-2 and IL-2Rα genes and of other cytokine genes, and upregulation of regulatory receptors such as CTLA-4, are also mostly identical to those seen in response to conventional antigens. Of interest, coligation of the TCR and CTLA-4 by SEE and B7 downregulates the response to bacterial SAg [56, 57], suggesting that the molecular mechanism(s) of CTLA-4 function is (are) operational on the TCR signalling cascades induced by SEE.

Alternative Coreceptors and Additional Receptors for Bacterial Superantigens

The ability of bacterial SAgs to activate T cells without using CD4/CD8 as coreceptors opens up the possibility that other cell surface receptors interact

with these toxins playing the role of a surrogate coreceptor and triggering the alternative signalling pathway described above. Indirect evidence supporting this idea comes from several laboratories. First, Arad et al. [58] have identified a homologous stretch of 12 amino acids in SEB, between residues 150 and 161 (TNKKKVTAQELD), that is relatively conserved among all bacterial SAgs and that is located in a domain separate from the TCR and MHC class II binding domains. A peptide containing such a sequence has antagonist activity against T cell responses to SAgs and blocks SAg-induced expression of IL-2, IFN-γ and TNF-α, cytokines that mediate shock. The presence of such a region, away from the interface facing the TCR and MHC molecules, and with functional repercussions raises the possibility of existence of a molecule acting as a coreceptor by binding to this sequence and potentially triggering downstream signalling pathways. We have detected an only partial effect by targeting lysine residue 147 in SEE (equivalent to K152 in SEB) suggesting that this charged residue could be involved in the binding to a molecule, other than the TCR and the class II MHC molecules [Bueno et al., unpubl. obs.]. However, the effect of the antagonist peptide mentioned above has not been reproduced on human MHC class II-triggered T cell responses [59].

Given the finding that the alternative TCR signalling pathway triggered by bacterial SAgs utilizes G proteins, it is logical to suggest that the coreceptor may be a G protein-coupled receptor. Among the many seven-transmembrane domain receptors, chemokine receptors are particularly attractive candidates. In this respect, Molon et al. [60], using an SEE-based experimental system with Jurkat T cells, have reported that CXCR4 and CCR5 can provide T cell costimulation through its association with G_{11} proteins. However, blockade of CXCR4 and CCR5 did not prevent SEE-induced T cell response by Lck-deficient Jurkat T cells [Bueno and Madrenas, unpubl. data]. Moreover, CXCR4 signalling requires Lck-dependent ZAP-70 phosphorylation [61, 62]. Thus, it is unlikely that these chemokine receptors are the ones responsible for primary triggering $G\alpha_{11}$-dependent PLC-β activation in an Lck-independent manner.

Membrane glycosphingolipids have also been reported to act as receptors for bacterial SAgs [63]. Among all the glycosphingolipids present in the membrane of T cells, GM1 is the most abundant glycosphingolipid, located preferentially in lipid rafts. Cross-linking of GM1 can trigger some signalling events including activation of ERK-1/2 [64–67]. GM1 is also present in all cell membranes and may provide a unifying molecule to explain the pleiotropic effects of bacterial SAgs.

Beyond the surrogate coreceptor idea, considerable evidence exists that the TCR β-chain and MHC class II molecules may not be the only functional receptors for bacterial SAgs. For example, because SEH was rigorously shown to activate T cells expressing human Vα10, whereas no changes in Vβ patterns

were seen, this SAg was proposed to interact directly with the TCR α-chain [97]. Also, the staphylococcal enterotoxins, but not TSST-1 or the streptococcal SAgs, are able to induce foodborne illness upon ingestion, yet the receptors and mechanism of this activity remain uncharacterized. Mutational analysis of SEA, SEB and SEC_1 have indicated that T cell activation was not required for emetic activity [68, 69] implying that TCR recognition is not necessary for foodborne illness. Furthermore, TSST-1 alone, in comparison with SEC_1 and SpeA, was shown to be able to cross mucosal surfaces, and it was hypothesized that this process may involve an unknown cellular receptor only recognized by TSST-1 [70].

T Cell Effector Responses Induced by Bacterial Superantigens

As indicated, TCR ligation by bacterial SAgs triggers T cell activation, leading to proliferation and cytokine production [71]. Later on, depending on the conditions of stimulation, some T cells undergo activation-induced cell death and those that survive become unresponsive and anergic [1, 72]. Both CD4+ T cells and CD8+ T cells respond to stimulation by SAg [73, 74]. However, changes in the binding affinity of SAgs to MHC haplotypes determine differences in the potency of those SAgs to induce T cell activation [75]. Although specific for each SAg, in general, the binding affinity of SAgs to HLA molecules follows this gradation: HLA-DR > HLA-DQ > HLA-DP [76]. This is also applicable to mouse MHC class II molecules, in which they bind with higher affinity to I-E (HLA-DR homologue) than to I-A (HLA-DQ homologue) [76]. In general, the binding affinity of SAgs to human MHC molecules is higher than to their mouse homologues, and that would explain the resistance of mice to develop toxic shock unless they are previously treated with D-galactosamine or lipopolysaccharide [76], or express human MHC class II molecules [77]. However, SEE, SEC_1, SEC_2 and SEC_3, but not SEA, SEB or SED, induce proliferative responses in MHC class II-deficient mice, although they require high doses of toxin and the potency of the response is low [79].

Upon SAg challenge in vivo, there is massive proliferation of the activated T cells and the number of Vβ-specific T cells increases dramatically. Later on, their number is reduced due to activation-induced apoptosis. This process involves the typical DNA fragmentation, and affects preferentially Vβ-specific CD4+ T cells [72]. Stimulation with specific antigen and proinflammatory mediators, such as lipopolysaccharide, rescues T cells from death and promotes survival of SAg-stimulated T cells [80, 81]. The mechanism that triggers T cell apoptosis after SAg challenge is not well understood, but several events can contribute to it: impaired IL-2 production, massive glucocorticoid release

following SAg administration in vivo and Fas/FasL signalling have all been shown to promote T cell deletion in response to SAgs [82–84]. Systemically, this process results in the 'hole' in the T cell repertoire observed in individuals affected by toxic shock syndrome and mice treated with SAgs [77, 85]. Such a 'hole' can lead to nonspecific immunosuppression, as it would deprive the organism of T cells potentially active against pathogens. Alternatively, it has been shown that SAg-mediated depletion of autoreactive T cells protects from the development of experimental models of autoimmunity such as murine collagen-induced arthritis, a model for human rheumatoid arthritis, and experimental autoimmune encephalomyelitis [86].

After the initial expansion, those SAg-stimulated T cells that survive become anergic and do not respond to secondary stimulation with the challenging SAg. Depending on the T cell function used as a readout, there are differences in the T cells that are involved: proliferation is suppressed in a Vβ-specific manner and affects preferentially CD4+ T cells, but inhibition of IL-2 production is not Vβ restricted [73, 87]. The anergic state can be maintained for several hours to days, depending on the SAg involved, the dose and the location [88]. CD28 costimulation reduces the anergizing effect of SAgs, as illustrated by the more pronounced hyporesponsiveness observed in CD28-deficient mice after SEB challenge [89]. A role for regulatory T cells has been shown in the SAg-induced anergy. Such regulatory cells can be CD4+CD25+ or CD4+ $CD25^-$ and their regulatory action involves cell contact-dependent suppression of APC activity [90, 91].

The effects of SAgs on T cell proliferation and anergy may be partially mediated by their effects on cytokine production. High levels of IL-2 are detected very early after SEA challenge in vivo, but IL-2 disappears very fast and it is absent by the time IL-2Rα expression is detected on T cells [82]. The production of other cytokines is differentially affected. In vitro, SAg stimulation of T cells induces massive production of the Th1-type proinflammatory cytokines, IFN-γ, TNF-α and TNF-β, but little Th2-type cytokine production, and addition of exogenous IL-4 and IL-10 reduces the production of the Th1 cytokines [92]. In vivo, the systemic production of TNF-α mediates the toxic effect of SAgs [93], through multiple processes including capillary leakage, hypotension and tissue necrosis [94]. During the course of SAg challenge in vivo, there is, however, production of IL-10 that protects from the lethal effects of TNF and IFN-γ in the murine models of SAg-induced toxic shock [95]. Despite the initial Th1 response to SAgs, persistent exposure to bacterial enterotoxins results in a downregulation of TNF and IFN-γ production. Depending on the SAg and the strain of mice, this downregulation is accompanied by an increase in IL-4 and/or IL-10 production that drives the development of an immunoregulatory response [95, 96].

Concluding Remarks and Future Avenues

Distinctive molecular aspects of T cell activation by bacterial SAgs will likely be revealed in the near future as we learn more about the structural nature of TCR-dependent signalling, and the spatial and temporal coordinates in which TCR signalling occurs. To predict just a few, we still need to confirm that the immunological synapses induced by bacterial SAgs do have the same architecture as those induced by peptide/MHC complexes. Are lipid rafts or any protein-protein interaction microdomains on the cell surface key in the initiation of signalling by bacterial SAgs? Are the principles of immune cell activation by bacterial SAgs applicable to activation of parenchymal cells? And if so, do they explain the symptoms seen in the course of SAg-associated diseases? Also, we do not know the degree of TCR oligomerization induced by SAgs, and the role, if any, that other molecules may play in this oligomerization. Identification of novel ligands for bacterial SAgs on the T cell surface may prove to be a key first step to start the characterization of the immunological synapse induced by bacterial SAgs. Ultimately, it remains a major challenge to identify the selective pressures on bacteria expressing SAgs, and how the selective pressures translate at the mechanistic level, particularly in the interaction between SAg-bearing bacteria and the human immune system. This knowledge may prove invaluable to prevent emerging diseases mediated by SAgs and, most importantly, to improve the management of SAg-associated diseases.

Note Added in Proof

Since submission of this paper, the work mentioned as 'Bueno et al., unpublished observation', has been published in Immunity 2006;25:67–78.

Acknowledgements

We thank our colleagues and members of the Madrenas and McCormick laboratories for helpful discussions and ideas. This work was supported by the Canadian Institutes of Health Research (CIHR), the Kidney Foundation of Canada, and the London Health Sciences Centre Multi-Organ Transplant Program. C.B. was supported by a fellowship from the Ontario Research and Development Fund, J.K.M. is a recipient of a CIHR New Investigator Award, and J.M. holds a Canada Research Chair in Transplantation and Immunobiology.

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Marone G (ed): Superantigens and Superallergens.
Chem Immunol Allergy. Basel, Karger, 2007, vol 93, pp 181–194

Modulation of Chemokines by Staphylococcal Superantigen in Atopic Dermatitis

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Abstract

Atopic dermatitis is a common pruritic and chronically relapsing inflammatory skin disease. Atopic dermatitis patients show disturbed skin barrier functions, frequent allergic responses against allergens, defects in the antimicrobial immune defense, and a genetic predisposition. Clinical and experimental evidence points to a role for staphylococcal superantigens during the initiation and amplification of atopic skin inflammation. In the past decade, numerous studies identified chemokines including CCL1, CCL2, CCL3, CCL4, CCL5, CCL11, CCL13, CCL17, CCL18, CCL20, CCL22, CCL26, CCL27 and CX_3CL1 to be associated with atopic dermatitis. Here we summarize recent findings suggesting a role for staphylococcal superantigens in the production of chemokines during the development of atopic skin inflammation.

Atopic Dermatitis

Atopic dermatitis (AD) is a chronic or chronically relapsing inflammatory skin disease with eczematous lesions demonstrating typical morphology and distribution, increased susceptibility to cutaneous infections, severe pruritus, elevated serum IgE, the presence of allergen-specific IgE, and peripheral blood eosinophilia [1]. The prevalence of AD rapidly increased during the past decades and is currently ranging between 10–20% in children and 1–3% in adults [1].

Clinical observations in AD combined with the results of recent scientific studies suggest an amplification cycle of atopic skin inflammation [2]. This cycle may start with pruritus representing a prominent symptom of AD [3]. Patients scratch and induce mechanical injury resulting in proinflammatory cytokine and chemokine production. Subsequently, chemokines in concert with an array of adhesion molecules direct the recruitment of pathogenic leukocytes to the skin [1, 4–6]. Within the skin, distinct leukocyte subsets may become activated via different pathways: (1) epithelial cell-derived cytokines (e.g. thymic stromal lymphopoietin) instruct dendritic cells to induce type 2 T helper (Th2) cell differentiation [7–11], (2) memory T cells encounter their specific antigen/allergen (e.g. Der p2 from house dust mites) or bacterial superantigens (e.g. staphylococcal enterotoxins) [1], (3) dendritic cells bind antigen/specific IgE complexes, capture antigen and show enhanced antigen presentation capabilities [1], (4) antigen/specific IgE complexes induce Fcε receptor aggregation and activate mast cells [1], and (5) viruses, fungi and bacteria take advantage of a reduced level of antimicrobial peptides present in AD skin, colonize inflamed skin and release proinflammatory products resulting in the modulation and amplification of leukocyte activation [12, 13]. As a common feature, leukocyte activation results in the release of inflammatory mediators including effector cytokines (IL-31) and proteases (tryptase), which together with stress-induced neuropeptides perpetuate pruritic signals [3, 14–16]. Taken together, these amplifying processes sustain inflammatory responses within the skin and lead to the development of an AD phenotype.

Notably, 90% of AD patients show lesions colonized with *Staphylococcus aureus* and exhibit a significant reduction of colonization in nonlesional skin [17–19]. Various products of *S. aureus* such as peptidoglycan and superantigens are known to induce the expression of proinflammatory cytokines in monocytes and dendritic cell subsets [20].

Clinical observations showing an improvement of patients following anti-staphylococcal therapy support a role for *S. aureus* in the pathogenesis of AD [21].

Chemokine Superfamily

Chemokines represent a family of small cytokine-like proteins, which has attracted significant interest with regard to understanding the mechanisms of leukocyte trafficking [22–24]. Chemokines are small (8–14 kDa) chemotactic peptides which organize innate as well as adaptive immune responses [24]. To date, 45 human chemokine ligands have been identified and this superfamily is thought to be among the first functional protein families completely characterized at the molecular level. On the basis of the arrangement of the amino-terminal

cysteine residues, chemokines can be divided into four subclasses: (1) CXC chemokines (termed as CXC ligands, CXCL), (2) CC chemokines (CCL), (3) C chemokines (XCL), and (4) CX_3C chemokines (CX_3CL) [23].

Chemokines interact with pertussis toxin-sensitive, 7-transmembrane-spanning G-protein-coupled receptors. To date, 10 CC chemokine, 7 CXC chemokine, 1 CX_3C and 1 XCR receptors have been characterized [23]. Chemokine receptor signaling triggers different intracellular pathways sustaining cell survival, inducing gene expression and most importantly enabling directional cell migration [22–24].

Interestingly, there is a certain degree of promiscuity in the chemokine superfamily with many ligands binding different receptors or vice versa. So-called 'cluster' chemokines representing chemotactic proteins which share a distinct chromosomal location are likely to bind the same receptors. However, 'noncluster or microcluster' chemokines are ligands which demonstrate a unique chromosomal location and tend to present a restricted or even specific chemokine receptor interaction [23].

Findings of recent studies show that in contrast to cluster chemokines, noncluster chemokines mediate nonredundant biological functions. CXCR4-CXCL12 interactions have been shown to play a crucial role in embryo-/organogenesis, guide hematopoietic stem cells into the bone marrow and are involved in the process of organ-specific metastases [25–29]. The expression of CXCR5 and CCR7 on distinct T, B and dendritic cell subpopulations directs their homing into peripheral lymph nodes [30–33], while CCR9 and CCR10 have been implicated in the tissue-specific trafficking of effector memory T cells into the gut or skin, respectively [5, 34, 35].

During the multistep process of leukocyte trafficking, chemokine ligand-receptor interactions mediate the firm adhesion of leukocytes to the endothelium and initiate transendothelial migration from the blood vessel into perivascular pockets [36]. From perivascular spaces, matrix-bound sustained chemokine gradients direct skin-infiltrating leukocyte subsets to subepidermal or intraepidermal locations. Hence, chemokines may be described as traffic signals guiding leukocytes through the organism. These chemotactic signals are crucial to sustain tissue homeostasis and initiate inflammation.

Chemokine Receptors and Th1 and Th2 Cells

For nearly a decade, the concept of type 1 and type 2 (Th1 and Th2) T cell responses has been applied to inflammatory diseases. Type 1 responses are initiated by IL-12 and characterized by T lymphocytes predominantly producing the effector cytokine IFN-γ. Conversely, type 2 T cell differentiation is driven

by IL-10 and prostaglandin E_2 and characterized by the production of IL-4, IL-5, and IL-13. Overall, atopic diseases have been associated with a Th2 phenotype showing dominance of IL-4, IL-5 and IL-13 secretion, blood eosinophilia, and elevated serum IgE levels. Recent observations in acute and chronic lesions of AD patients together with kinetic studies using the atopy patch test indicate that although the initiation of atopic skin lesions is driven by IL-4-producing Th2 cells, chronic lesions show either the coexistence of both IL-4-producing type 2 and IFN-γ-producing Th1 cells, or Th1 dominance [1]. Furthermore, the presence of IFN-γ-producing T cells correlates with the chronification and severity of AD skin lesions [1].

Since the discovery of chemokine receptors, considerable emphasis has been directed to the characterization of the receptor repertoire of polarized Th1 and Th2 cells, respectively [37–43]. Although there have been extensive discussions, it is now widely agreed that chemokine receptors are not exclusively expressed on either T lymphocyte subset. However, CXCR3 appears to be expressed by the majority of type 1 cells while only a minor population of type 2 cytokine-producing lymphocytes will express this receptor [43]. Conversely, CCR4 has been found on the cell surface of the majority of Th2 cells [43]. For other chemokine receptors, including CCR8, CCR5 and CCR3, the situation remains less clear. Furthermore, it is important to realize that in patients, we may not find the same highly polarized lymphocyte subsets generated in vitro and that the inflammatory infiltrate is likely to be composed of a mixture of both Th cell subsets with probably one dominating the other depending on the disease.

Association of Chemokines with an Atopic Dermatitis Phenotype

In the past decade, numerous studies identified chemokines associated with AD. These chemokines include CCL1, CCL2, CCL3, CCL4, CCL5, CCL11, CCL13, CCL17, CCL18, CCL20, CCL22, CCL26, CCL27, and CX_3CL1 [5, 44–70]. Notably, serum levels of CCL11, CCL17, CCL18, CCL22, CCL26, CCL27 and CX_3CL1 directly correlated with disease severity suggesting an important role in the immunopathogenesis of AD. Among these chemokines, CCL1, CCL17, CCL18, CCL22 and CCL27 are likely candidates to critically regulate the recruitment of memory T cells to sites of atopic skin inflammation. Moreover, serum levels of CCL11, CCL17, CCL22, CCL26, CCL27 and CX_3CL1 directly correlated with disease activity suggesting an important role in the pathogenesis of AD.

Patients suffering from AD show increased CCL27 protein production within the epidermis and the vast majority of skin-infiltrating lymphocytes (>90%) express CCR10 [5]. In vivo, intracutaneous injection of CCL27

induced the accumulation of CD3+ T cells [5]. Conversely, neutralization of CCL27-CCR10 interactions impaired memory T cell recruitment to the skin and suppressed allergen-specific skin inflammation in mouse models mimicking allergic contact dermatitis and AD [5].

Th2 cell lines and clones isolated from lesional skin of AD patients abundantly express CCR4 and show little or no CCR3, CCR8 and CXCR3 on their cell surface. To date, there are two known ligands for CCR4, CCL17 and CCL22 [23]. CCL17 and CCL22 are produced by different cell types. In humans, the major sources of CCL17 in the skin are dermal endothelial cells and keratinocytes, whereas CCL22 is secreted by macrophages, interstitial dendritic cells, and epidermal Langerhans cells. Hence, CCL17 expressed by the dermal endothelial cells and infiltrating dermal cells of atopic lesional skin may act during the first steps of T cell recruitment by inducing integrin-dependent adhesion and transendothelial migration of T cells while CCL22 supports the formation of T cell-dendritic cell clusters at sites of atopic skin inflammation.

A recent study by Reiss et al. [6] suggested that ligands of CCR4 and CCR10 cooperate in the recruitment of memory T cells to sites of skin inflammation. According to this model, CCL17 displayed by cutaneous venules, in combination with other CCR4 ligands trigger the integrin-dependent arrest and extravasation of lymphocytes rolling on cutaneous venules. Subsequently, CCL27, highly and selectively expressed by keratinocytes, may support diapedesis and epidermotropism of skin-homing T cells [5, 6].

Although the role of CCL17 and CCL27 during the recruitment of memory T cells is well established, both chemokines do not show a specific association with an AD phenotype but are expressed and potentially play a role in most chronic inflammatory skin diseases. Interestingly, recent studies suggest a link between the increased susceptibility of atopic individuals to bacterial skin colonization, the production of bacterial superantigens and regulation of chemokines.

Staphylococcal Superantigens Induce Chemokine Production in Atopic Dermatitis Patients

Antimicrobial peptides such as cathelicidins (LL-37) and β-defensins represent another integral component of the innate immune system of human skin [13, 71, 72]. The combination of cathelicidins and human β-defensin-2 shows synergistic antimicrobial activity against *S. aureus* [13]. Recently, a new antimicrobial peptide, HBD-3, has been identified which has potent killing activity against *S. aureus* by itself and can kill a broad spectrum of microbes [71, 73].

Two recent studies demonstrated that the presence of antimicrobial peptides is significantly decreased in acute and chronic lesions of AD patients [13, 74].

In vitro, IL-4 as well as IL-13 are potent inhibitors of TNF-α- and IFN-γ-induced human β-defensin-2 and -3 expression [13, 74].

Hence the deficiency of antimicrobial peptide production may account, at least in part, for the susceptibility of patients with AD to skin infections, promote bacterial colonization (e.g. *S. aureus*) and may, through the exposure to microbial products (e.g. superantigens, peptidoglycan, lipoteichoic acid), significantly contribute to the amplification of atopic skin inflammation.

Murine Studies

Recently, two independent studies have investigated the role of epicutaneous staphylococcal enterotoxin B (SEB) exposure in the development of skin inflammation. Laouini et al. [75] determined whether epicutaneous sensitization of mice with SEB results in allergic skin inflammation. BALB/c mice were epicutaneously sensitized with SEB, which elicited a cutaneous, inflammatory response characterized by dermal infiltration with eosinophils and mononuclear cells and increased mRNA expression of the Th2 cytokine IL-4 but not of the Th1 cytokine IFN-γ [75]. Epicutaneously sensitized mice mounted a systemic Th2 response to SEB resulting in elevated total and SEB-specific IgG1 and IgE [75]. Although epicutaneous sensitization with SEB resulted in selective depletion of SEB-specific T cell receptor Vβ8+ cells from the spleen and sensitized skin, splenocytes from SEB-sensitized mice secreted relatively more IL-4 and less IFN-γ than did saline-sensitized controls [75]. Taken together, the authors concluded that epicutaneous exposure to superantigens skews the immune response toward Th2 cells, leading to allergic skin inflammation and increased IgE synthesis that are characteristic of AD.

In contrast to Laouini et al. [75], a recent study by Savinko et al. [76] investigated the role of epicutaneous SEB in the modulation of allergen [ovalbumin (OVA)]-induced skin inflammation. Mice were topically exposed to SEB, OVA, a combination of OVA and SEB (OVA/SEB), or saline alone. Topical SEB as well as OVA/SEB exposure induced epidermal accumulation of CD8+ T cells and T cell receptor Vβ8+ cells in contrast to OVA application, which induced a mainly dermal infiltration of CD4+ cells [76]. SEB and OVA/SEB exposure elicited a mixed Th1/Th2-associated cytokine and chemokine expression profile within the skin. CCL3 and CCL4 mRNA levels were strongly elevated following OVA/SEB exposure compared to OVA challenge alone. Furthermore, OVA/SEB exposure also had synergistic effects on the expression of other proinflammatory chemokines including CCL1, CCL11 and CCL17 compared to epicutaneous treatments with allergen or SEB alone [76].

Next to these Th2-associated chemokines, Savinko et al. [76] also observed that the expression of Th1-associated and IFN-γ-inducible chemokines (CXCL9, CXCL10) as well as their corresponding receptor CXCR3 is highly

upregulated in OVA/SEB-exposed skin compared to OVA sensitization alone. Taken together, topical exposure of mice to SEB provokes the production of a distinct set of cytokines and chemokines leading to epidermal accumulation of CD8+ T cells, a mixed Th2/Th1 type dermatitis and vigorous production of specific IgE and IgG2a antibodies, which can be related to the chronic phase of atopic skin inflammation.

Human Studies

A systematic analysis of the expression of all known chemokines in chronic inflammatory skin diseases identified CCL18 to be specifically associated with an AD phenotype but absent in other chronic inflammatory or autoimmune skin diseases such as psoriasis or cutaneous lupus erythematosus [60]. Among all known chemokines, CCL18 represented the most highly expressed ligand in AD and the absolute amount of CCL18 mRNA in lesional atopic skin was more than 100-fold higher than that seen for CCL17 [60]. In good accordance with this finding, a DNA microarray screen also identified CCL18 as one of the genes showing the strongest association with AD compared to psoriatic or normal skin specimen [12]. Pivarcsi et al. [60] demonstrated that allergen exposure and staphylococcal superantigens markedly induced CCL18 expression in vitro and in vivo suggesting important CCL18-driven processes during the initiation and amplification of atopic skin inflammation. In analogy to AD patch tests, CCL18 expression was examined in AD patients before as well as 2, 6 and 48 h after patch testing with the superantigen SEB. Topical exposure to SEB induced skin lesions clinically and histopathologically resembling AD, and subsequent quantitative real-time PCR analyses showed that the expression of CCL18 was significantly induced in skin samples 48 h after epicutaneous exposure to SEB. Furthermore, superantigen (SEB) stimulation of peripheral blood mononuclear cells resulted in a rapid (6 h) and marked increase in CCL18 expression in vitro suggesting that skin-infiltrating leukocytes are the major source of superantigen-triggered CCL18 production [60].

Although its receptor is yet unidentified, CCL18 bound to the surface of CLA+ skin-homing memory T cells and induced the migration of memory T cells into human skin, in vivo [61]. CCL18 was produced by dermal dendritic cells in close proximity to infiltrating T cells implicating a role in the formation of T cell-dendritic cell contacts with atopic skin [60].

A recent report by Gombert et al. [44] demonstrated that the expression of the CC chemokine CCL1 is also significantly and selectively upregulated in AD in comparison to psoriasis, cutaneous lupus erythematosus or normal skin. The CCL1 gene is located in a chemokine gene cluster including 15 different ligands on chromosome 17q11.2. Many of the CC chemokines in this cluster bind the same receptors, namely CCR1, CCR2, CCR3 and CCR5. Interestingly,

while the gene encoding CCL1 is located in this chemokine gene cluster, it is the only CC chemokine from this cluster that specifically binds CCR8, in other words, it has a single ligand/receptor relationship. This suggests that CCL1-CCR8 interactions may mediate nonredundant functions.

Gombert et al. [44] reported that CCL1 serum levels of AD patients were significantly higher than those of healthy individuals. Dendritic cells, mast cells and dermal endothelial cells were identified as abundant sources of CCL1 during atopic skin inflammation and allergen challenge as well as *S. aureus*-derived superantigen (SEB) significantly induced its production [44]. In vitro, binding and cross-linking of IgE on mast cells resulted in a significant upregulation of this inflammatory chemokine [44]. Its specific receptor, CCR8, was expressed on a small subset of circulating T cells and was abundantly expressed on interstitial dendritic cells, Langerhans cells generated in vitro, and their monocytic precursors. Although dendritic cells maintained their CCR8+ status during maturation, brief activation of circulating T cells recruited CCR8 from intracytoplasmic stores to the cell surface [44]. Moreover, the inflammatory and atopy-associated chemokine CCL1 synergized with the homeostatic chemokine CXCL12 resulting in the recruitment of T (CD8 > CD4) and Langerhans cell-like dendritic cells [44]. CCL1 has also been described as a potent antiapoptotic factor for thymocytes, suggesting that CCL1-CCR8 interactions may provide survival signals for T cells and dendritic cells at sites of atopic skin inflammation.

These findings suggest that, in atopic individuals, staphylococcal superantigens may trigger CCL1 and CCL18 production which in turn recruit memory T cells and in particular Langerhans cells into the skin leading to their accumulation in subepidermal and intraepidermal locations. Since activated dendritic cells secrete large amounts of CCL1, CCL1-CCR8-driven recruitment pathways may facilitate dendritic cell-T cell interactions at sites of atopic skin inflammation. Moreover, CCR8 signalling may enhance cell survival providing a potential mechanism that sustains atopic inflammation and prevents activation-induced apoptosis of skin-infiltrating leukocytes. Thus, CCL1 and CCL18 may provide pathways linking adaptive and innate immune functions, leading to the accumulation of relevant leukocyte subsets at sites of atopic skin inflammation and supporting the initiation and amplification of AD.

Until recently, the contribution of staphylococcal superantigen and skin-infiltrating effector memory T cells to the development of pruritus remained an enigma. A recent study by Dillon et al. [14] demonstrated that transgenic overexpression of the novel 4-helix bundle cytokine IL-31 in lymphocytes induces severe pruritus and dermatitis in mice. IL-31 is preferentially expressed by Th2 cells and signals through a heterodimeric receptor composed of IL-31 receptor A and oncostatin M receptor [14]. Subsequently, studies by Bilsborough et al. [77] and Sonkoly et al. [78] extended these observations and showed that

human IL-31 is significantly upregulated in 'pruritic' (AD and prurigo nodularis) but not in 'nonpruritic' (psoriasis) forms of chronic skin inflammation. Within the inflammatory infiltrate of patients with AD, inflammatory cells of the lymphocytic lineage, with the majority staining for CLA and CD3, overexpressed IL-31 mRNA [77]. In vivo exposure to staphylococcal superantigen rapidly induced IL-31 expression in atopic individuals [78]. In vitro, IL-31 was induced by staphylococcal superantigens but not by Th1- or Th2-type cytokines or viruses in leukocytes [78]. In atopic individuals, activated leukocytes expressed higher levels of IL-31 transcripts compared with those seen in healthy nonatopic subjects [78]. These observations were in line with findings of Bilsborough et al. [77], showing that circulating CLA+ memory T cells of patients with AD were capable of producing increased levels of IL-31 protein when compared with T cells of psoriatic donors. In addition, IL-31 stimulated human primary keratinocytes to produce a distinct set of chemokines including CCL1, CCL4, CCL17, CCL19, CCL22 and CXCL1 [14].

Conclusions

Results of recent studies suggest that staphylococcal superantigens may enhance chemokine production through several different pathways (fig. 1). First, superantigens may directly upregulate the expression of a distinct set of chemokines in leukocytes. Second, they may indirectly promote the release of effector cytokines, including IL-4, IFN-γ or IL-31, from memory T cells and in turn enhance the production of chemokines in surrounding structural cells of the skin. Third, superantigen-induced IL-31 production causes severe pruritus and subsequent trauma through scratching leading to the release of proinflammatory cytokines which in turn may amplify chemokine expression.

Taken together, the colonization with *S. aureus* and the release of bacterial superantigens may represent an important trigger factor in the complex amplification cycle of atopic skin inflammation. Hence, antiseptic strategies as well as modalities that enhance endogenous antimicrobial defense in atopic individuals may represent useful tools to support the therapeutic management of patients suffering from AD.

On the other hand, novel chemokine receptor antagonist-based strategies to interfere with skin inflammation are likely to be preventive rather than therapeutic. Chemokine receptor antagonists hold promise to provide excellent tools to impair the recruitment of pathogenic leukocyte subsets to the skin or other peripheral sites. Once leukocytes have entered their target organ and undergone activation processes, chemokine antagonists are likely to be less effective. Together with the availability of potent drugs to treat atopic skin inflammation

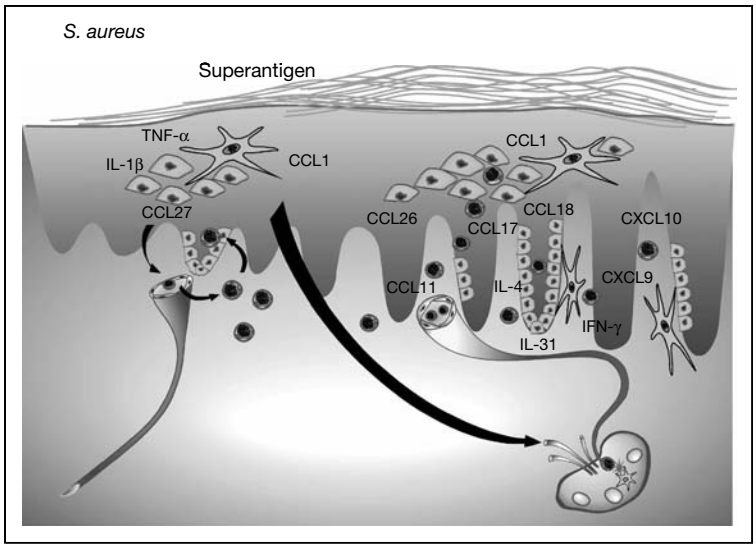

Fig. 1. Staphylococcal superantigens modulate chemokine production in AD. First, superantigen exposure may directly lead to the production of chemokines by T cells and dendritic cells (CCL1, CCL18). Second, superantigens may induce the release of effector cytokines such as IL-4, IFN-γ or IL-31 which in turn may upregulate the expression of CCL1, CCL11, CCL17, CCL18, CCL26, CXCL9 or CXCL10. Third, IL-31-induced pruritus may be accompanied by skin injury through scratching resulting in the production of primary proinflammatory cytokines such as IL-1β and TNF-α which in turn may amplify chemokine production (e.g. CCL20, CCL27).

like glucocorticosteroids, topical immunomodulators (tacrolimus, pimecrolimus) or cyclosporine, chemokine receptor antagonists may represent promising candidates to reduce the frequency of acute flares, prolong the lesion-free interval and provide optimized long-term management of patients suffering from chronically relapsing inflammatory skin diseases such as AD.

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Marone G (ed): Superantigens and Superallergens.
Chem Immunol Allergy. Basel, Karger, 2007, vol 93, pp 195–213

Role of Superallergens in Allergic Disorders

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Abstract

A significant percentage of allergic diseases (e.g. certain cases of intrinsic asthma, chronic idiopathic urticaria, and atopic dermatitis) cannot be explained by the classical mechanisms of IgE/allergen-mediated activation of basophils and mast cells. We found that protein Fv, an endogenous protein synthesized in the human liver and increased during viral hepatitis, act as a superallergen by binding to IgE of the V_H3 family and activating human basophils and mast cells. Similarly, envelope gp120 of HIV-1 and protein A of *Staphylococcus aureus* are viral and bacterial superallergens, respectively, because they interact with IgE V_H3+. Protein L binds to the V domain of he κ light chains of IgE. Our results demonstrate that endogenous, viral and bacterial products activate primary effector cells of allergic disorders to release proinflammatory mediators and cytokines thereby acting as immunoglobulin superantigens (superallergens).

Allergic disorders comprise a heterogeneous spectrum of diseases that manifests as bronchial asthma, rhinosinusitis, anaphylaxis, urticaria and atopic dermatitis, and food allergy. Allergies in one form or another affect a large part of humanity and are associated with substantial morbidity, mortality, and economic cost [1]. The prevalence of asthma and allergic disorders has risen steadily this century in westernized societies, doubling in the past 20–40 years [2, 3]. Allergic diseases are the results of complex interactions between genetic and environmental factors [4–6].

There is compelling evidence that several distinct phenotypes of asthma and other allergic disorders exist [7]. In particular, atopic asthma, atopic dermatitis and food allergies are caused by the immune reaction to common proteins, known as allergens. These proteins appear harmless in themselves, and only cause disease when they stimulate the production of IgE that binds to its high-affinity receptor (FcεRI) present on tissue mast cells and peripheral blood basophils (FcεRI+ cells) [8]. Exposure to a specific allergen in a sensitized individual leads to cross-linking of IgE/FcεRI complexes, which causes the release of proinflammatory mediators, cytokines, and chemokines from FcεRI+ cells and the initiation of allergic inflammation [9, 10].

Conventional antigens usually stimulate less than 0.001% of naive lymphocytes. By contrast, superantigens can stimulate more than 5% of the naive lymphocyte pool [11]. This immunologic property stems from the unique ability of superantigens to interact specifically with most lymphocytes that express antigen receptors from a particular variable (V) region gene family. Binding of a superantigen involves antigen receptor sites in the V regions that are common to members of the V gene family, but that are spatially distinct from the complementary-determining region sites implicated in antigen binding. Classical superantigens are T cell superantigens (staphylococcal enterotoxins and toxic shock syndrome toxin-1).

Some naturally occurring proteins possess the properties of superantigens for B lymphocytes. B cell superantigens are proteins endowed with immunoglobulin-binding capacities that parallel the lymphocyte-activating properties of T cell superantigens. The best characterized of these immunoglobulin superantigens is *Staphylococcus aureus* protein A which is the prototype of a B cell superantigen [12]. Other B cell superantigens are the gp120 envelope glycoprotein of HIV-1 [13], protein L from *Peptostreptococcus magnus* [14] and a human gut-associated sialoprotein termed 'protein Fv' [15].

The concept of immunoglobulin superantigens applied to the pathophysiology of allergic disorders led to the definition of 'superallergens' to indicate proteins of various origins able to activate FcεRI+ cells by interacting with membrane-bound IgE [16].

Figure 1 shows the four canonical mechanisms by which cross-linking of the IgE-FcεRI network induces mediator release from human mast cells and basophils [8, 10]. A multivalent antigen can cross-link at least two specific IgE molecules bound to FcεRI+ cells to release mediators [17]. Anti-human IgE antibodies (anti-IgE) possess two binding sites for the Fcε region of human IgE and are potent stimulators of histamine and cytokine secretion from these cells [18]. Similarly, antibodies directed against epitopes of the α-chain of human FcεRI (anti-FcεRIα) can activate human FcεRI+ cells [19]. Finally, immune complexes containing IgG against IgE can activate human basophils [20].

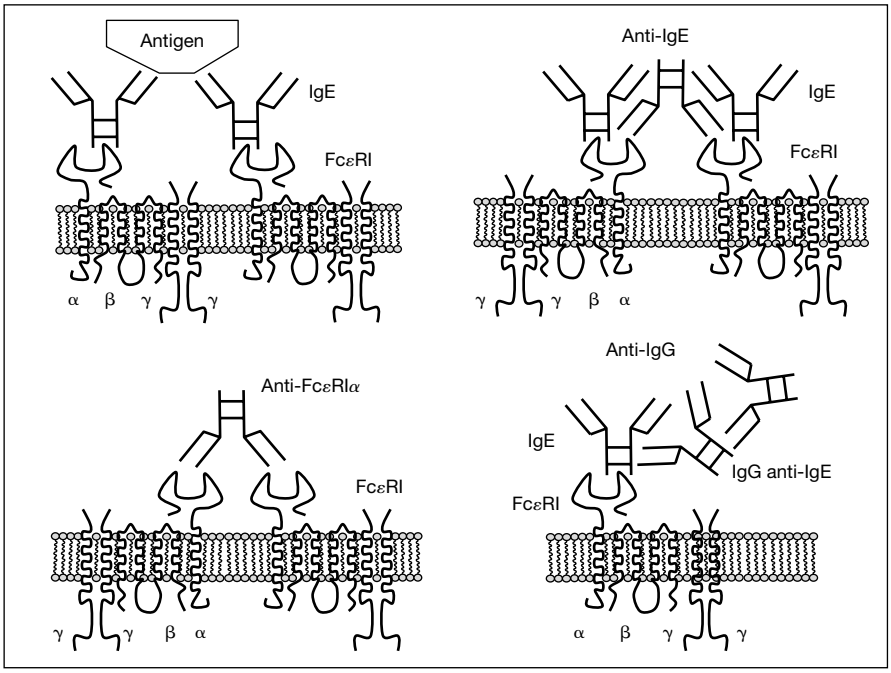

Fig. 1. Schematic representation of the four canonical mechanisms by which cross-linking the IgE-FcεRI network can induce the release of mediators from human basophils and mast cells. A multivalent antigen can cross-link at least two specific IgE molecules bound to FcεRI+ cells to release mediators. Anti-IgE antibodies possess two binding sites for the Fcε region of human IgE and activate mediator secretion from these cells. Antibodies directed against an epitope of the α-chain of FcεRI (anti-FcεRIα) can also trigger the release of mediators. There is also some evidence that immune complexes containing IgG anti-IgE and anti-IgG can activate human basophils.

It is generally believed that these four canonical mechanisms of IgE-mediated activation of human FcεRI+ cells are responsible for the pathophysiologic involvement of these cells in allergic disorders [7]. However, a significant percentage of allergic diseases (e.g. certain cases of asthma, chronic idiopathic urticaria, and atopic dermatitis) cannot be explained by the four classical mechanisms of FcεRI+ cell activation.

During recent years, we have evaluated the hypothesis that immunoglobulin superantigens (superallergens) of various origins (endogenous, viral and bacterial) can activate human basophils and mast cells to release proinflammatory mediators and cytokines.

Protein Fv Is an Endogenous Superallergen That Activates Human Basophils and Mast Cells

Protein Fv is a 175-kDa dimeric sialoprotein produced in the human liver and released in biological fluids during viral hepatitis [21], which possesses unique immunoglobulin-binding activity [22]. It occurs as a free molecule in the gut lumen of patients with hepatitis A, B, C, and E [23] and in monkeys experimentally infected with HCV and E viral hepatitis [23, 24]. Protein Fv binds to the V domain of the heavy (H) chains of immunoglobulin, irrespective of immunoglobulin class, subclass, and light (L) chain type [25]. A single protein Fv molecule can bind six $F(ab')_2$ fragments [22] of human IgM, IgG, and IgE [21, 22, 25]. Binding of protein Fv to the V_H region of immunoglobulins occurs in a domain external to the conventional antigen-binding pocket [21, 25].

Acute and chronic viral hepatitis infections are associated with many diverse extrahepatic tissue injuries, not all of which are directly related to the cytotoxic effects of HBV and HCV. In fact, skin rashes and urticarial reactions occur in about 5% of patients with viral hepatitis [26–28].

We asked whether protein Fv from HCV-infected patients can release histamine from basophils and mast cells purified from human lung and skin tissues. To this aim, we compared the histamine-releasing activity of increasing concentrations of protein Fv from patients with HCV infection and anti-IgE on histamine release from human basophils and mast cells. Our experiments showed that the concentration-response curves induced by protein Fv paralleled those induced by anti-IgE [19]. However, protein Fv was approximately 100 times more potent than anti-IgE. The same preparations of protein Fv absorbed with protein A-Sepharose coated with human polyclonal IgG lost their immunoglobulin-binding capacity and did not induce histamine release [19, 29]. Consequently, protein Fv was responsible for secretion of the mediator from human FcεRI+ cells, presumably by binding to immunoglobulins. Protein Fv functioned as a complete secretagogue because it also induced the de novo synthesis of prostaglandin D_2 and cysteinyl leukotriene C_4 by lung mast cells [29].

We next examined the relationship between protein Fv and IgE by using cross-desensitization between these two stimuli. Human basophils and mast cells 'desensitized' to protein Fv released approximately 90% less histamine when challenged with anti-IgE. Similarly, when cells pretreated with protein Fv were challenged with anti-IgE, they lost their releasing ability with the heterologous stimulus. This finding suggests that the releasing property of protein Fv occurs mainly through interaction with IgE on the basophil or mast cell surface. Additional evidence that protein Fv induces histamine release by binding to IgE comes from the observation that protein Fv does not induce mediator release from basophils or mast cells stripped of IgE from FcεRI by brief exposure to lactic acid [19, 29].

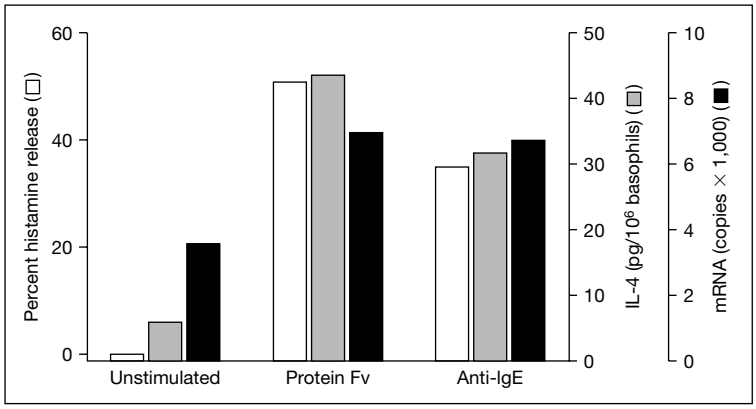

Fig. 2. Effects of anti-IgE and protein Fv on the intracellular levels of IL-4 mRNA copies, on the extracellular protein levels of IL-4, and on the release of histamine from basophils compared with unstimulated cells. (Reproduced with permission from Patella et al. [19].)

Protein Fv binds specifically to the V_H domain of immunoglobulins [22]. Consequently, this endogenous protein is similar to multivalent antigens or to divalent anti-IgE antibodies. To evaluate the mechanism whereby this protein activates basophils, we incubated protein Fv with human monoclonal IgM from diverse V_H families [19, 29]. Preincubation of basophils with three preparations of human monoclonal IgM V_H3+ concentration-dependently inhibited the histamine-releasing activity of protein Fv. In contrast, a monoclonal IgM that has a V_H6 domain had no such effect. These results are compatible with the hypothesis that protein Fv binds to IgE V_H3+ bound to FcεRI+ cells.

In conclusion, protein Fv, an endogenous protein present in normal liver and released in biological fluids during viral hepatitis [21, 22], is a potent inducer of the release of proinflammatory mediators from human FcεRI+ cells. This was the first evidence that a human protein induced by viral infections can act as an endogenous immunoglobulin superantigen. In fact, protein Fv functions as an endogenous superallergen and reproduces the releasing activity of specific antigens and of anti-IgE on FcεRI+ cells [19, 29].

We have also demonstrated that low concentrations of protein Fv induce IL-4 secretion from basophils [19]. A study in which we examined IL-4 expression in resting basophils and in protein Fv-stimulated basophils demonstrated that protein Fv induced an increase in IL-4 similar to that obtained with anti-IgE (fig. 2). The stimulation of IL-4 synthesis in basophils by protein Fv was mediated by interaction with IgE. In fact, brief exposure to lactic acid completely blocked the effect of protein Fv and of anti-IgE on IL-4 secretion from

basophils. Preincubation of basophils with preparations of monoclonal IgM, possessing a V_H3 domain [19], inhibited the IL-4-releasing activity of protein Fv. These results demonstrate that protein Fv is a potent inducer of IL-4 synthesis and release mediated by interaction with the V_H3 region of IgE present on basophils.

This was the first evidence that an endogenous superallergen, protein Fv, released during viral infections, can induce the synthesis and secretion of such an important cytokine as IL-4 from basophils. The activity of IL-4 has been linked to several immunoregulatory functions, e.g. the synthesis of IgE by B lymphocytes, and it is intriguing that some patients with viral hepatitis have high serum IgE levels [30, 31].

The in vitro activities of protein Fv are very potent, which suggests that it could exert relevant effects in vivo. It is feasible that protein Fv released in the blood of patients with acute and chronic HCV, HAV or HBV infection contributes, through the release of mediators from FcεRI+ cells, to the allergic manifestations observed in some of these patients [26–28].

The mechanism whereby protein Fv activates FcεRI+ cells represents a new pathogenetic cascade, namely, viral infection, endogenous superallergen production, activation of human FcεRI+ cells and tissue injury. In a more general context, this raises the possibility that other endogenous immunoglobulin superantigens induced by viruses can cause tissue injury through this mechanism that involves human FcεRI+ cell activation.

Envelope gp120 of HIV-1 Is a Viral Superallergen Activating Human FcεRI+ Cells through Interaction with IgE V_H3+

The last decades of the 20th century saw the emergence of the acquired immunodeficiency syndrome (AIDS) due to the human immunodeficiency viruses (HIV-l and HIV-2) [32] and the pandemic of allergic disorders [1–3]. These two immunological disorders do not appear to be related, AIDS being an acquired immunodeficiency of viral etiology [32], whereas atopic diseases resulting from overproduction of IgE in response to allergens [7]. Nevertheless, these disorders share several features. For instance, atopic disorders are characterized by elevated serum IgE levels and there is also an increase in IgE levels in HIV-1-infected children [33–37] and adults [38–42]. The helper T cell (Th) type 1 and 2 cytokine profile is deranged in HIV-1 infection because of a shift towards Th2 cytokines [43–46]. It is noteworthy that HIV-1-infected patients have an increased incidence of allergic disorders [47, 48].

HIV-1 enters immune cells via viral envelope glycoproteins that are organized in oligomeric, probably trimeric, spikes on the virion surface. The spike

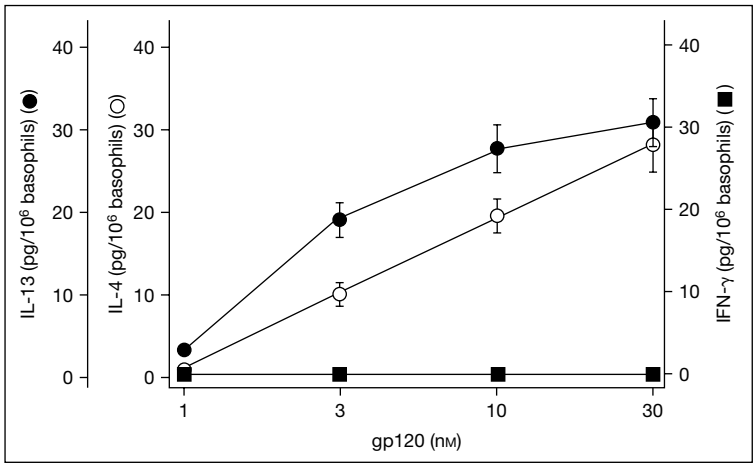

Fig. 3. Effects of various concentrations of gp120 on IL-4, IL-13 and IFN-γ secretion from human basophils obtained from normal donors. Purified basophils were incubated with gp120 for 4 h at 37°C (IL-4) or 18 h at 37°C (IL-13 and IFN-γ). Each point represents the mean ± SEM obtained from four experiments. Error bars are not shown when graphically too small. (Reproduced with permission from Marone et al. [16].)

surface consists of gp120 associated with gp41 via noncovalent interaction [49]. Entry of HIV-1 viruses into immune cells involves gp120 binding to the CD4 glycoprotein, which serves as a primary receptor [50]. CD4 binding results in conformational changes in gp120, namely, the exposure and/or formation of binding sites for chemokine receptors that serve as obligate coreceptors for virus entry [51]. Interaction with coreceptors results in other conformational changes in the envelope protein and exposure of the hydrophobic fusion domain of the gp41 subunit, which, in turn, mediates fusion of the opposed cell and virus membranes [50].

HIV-1 enters the body mainly through mucosal surfaces, which contain an abundance of mast cells. Moreover, early infection is associated with a high level of viremia [32]. Thus, tissue mast cells and peripheral blood basophils can be exposed to shed or virus-bound gp120 during the early phases of infection. This raised the question as to whether HIV-1 gp120 can activate human FcεRI+ cells from donors negative for HIV-1 and HIV-2 antibodies. HIV-1 gp120 is an immunoglobulin superantigen [52, 53] and immunoglobulin V_H3 gene products are the ligand for gp120 [54]. Therefore, we investigated whether HIV-1 gp120 interacts with the V_H3 domain of IgE to induce the release of proinflammatory mediators and cytokines from human FcεRI+ cells. Our results showed that gp120 stimulated IL-4 and IL-13 release from basophils [55, 56] (fig. 3). In

contrast, IFN-γ mRNA was absent from all gp120-stimulated basophil preparations. This demonstrates that gp120-mediated stimulation of FcεRI+ cells induces only Th2-like cytokines.

We also compared four recombinant gp120 ($gp120_{MN}$, $gp120_{SF2}$, $gp120_{LAV}$ and $gp120_{EM}$) derived from divergent HIV-1 isolates obtained from different viral clades (B and E) of various geographical origins. These divergent gp120 samples induced IL-4 and IL-13 release from basophils, which implies that the capacity to induce Th2-like cytokine release from basophils is a feature of gp120 maintained throughout the evolution of HIV-1.

Brief exposure to low pH removes most of the IgE bound to FcεRI+ cells and completely blocks the effects exerted by gp120 and by anti-IgE on IL-4 and IL-13 secretion from basophils [55, 56]. This indicated that gp120-induced activation of FcεRI+ cells occurs via the interaction with basophil-bound IgE [57]. Preincubation of gp120 with human monoclonal IgM V_H3+ inhibited the effect of gp120 on cytokine secretion from basophils. In contrast, preincubation with a monoclonal IgM V_H6+ had no effect. Thus, binding to the V_H3 domain prevents gp120 interaction with IgE bound to FcεRI on basophils and mast cells.

The demonstration that gp120 from diverse clades induces the synthesis and release from human FcεRI+ cells of two cytokines critical for Th2 polarization suggests that during early HIV-1 infection, associated with high levels of viremia [32], this process may serve to provide an initial source of IL-4 and IL-13, which would favor a shift toward a Th2 phenotype [58]. In advanced HIV-1 infection, when CD4+ T cells are decreased, FcεRI+ cells might also provide a relevant pool of Th2-like cytokines.

The V_H3 family is the largest in the human cell repertoire (approx. 50%) [52, 53], therefore shed or virus-bound gp120 could interact with high frequency with IgE V_H3+ bound to basophils of individuals at an early stage of infection. Consequently, the viral superantigen gp120 can rapidly activate FcεRI+ cells via IgE V_H3+.

The prevalence and/or severity of allergic reactions and adverse reactions to drugs are enhanced in patients with HIV-1 infection [47, 48]. Similarly, IgE levels can be increased in HIV-1 infection in children [33–37] and adults [38–42]. Elevated IgE levels have been associated with the progression of HIV-1 disease [33–37, 42, 59], which suggests that increased IgE levels could indicate a poor prognosis in patients with HIV-1 infection [42, 59]. Do these seemingly unrelated clinical and biological findings stem from a single biological and immunological substrate? It is conceivable that gp120 functions as a viral superantigen by interacting with IgE V_H3+ to activate human FcεRI+ cells. This might explain the elevated IgE levels and increased prevalence of allergic reactions associated with HIV-1 infection.

Protein A Is a Staphylococcal Superallergen That Activates Human FcεRI+ Cells by Interacting with IgE V_H3+

Most clinical isolates of *S. aureus* synthesize protein A, a cell wall protein endowed with unique immunoglobulin-binding properties. Protein A has a classical site that binds the Fcγ of IgG [60], and an alternative site that binds the Fab portion of 15–50% of human polyclonal IgG, IgM, IgA, and IgE [61].

S. aureus, found in the skin lesions of approximately 90% of patients with chronic atopic dermatitis, might be an important trigger factor in the inflammatory process [62–64]. There is also evidence that inflammatory processes as consequences of microbial infections play a role in certain forms of allergic rhinitis and nasal polyps [65, 66]. In addition, it has been proposed that *S. aureus* plays a role as a trigger factor in certain forms of asthma, and IgE against *S. aureus* toxins was found more frequently in patients with severe asthma than in controls [67]. Moreover, an increased number of T cells expressing the T cell receptor β-chain V region, known to be induced by microbial superantigens, was detected in bronchial lavage fluid of asthmatics [68, 69].

We asked whether protein A and *S. aureus* Cowan 1, a strain that synthesizes protein A, can release proinflammatory mediators from basophils and mast cells. We found that both *S. aureus* Cowan 1 and soluble protein A increased histamine release from human basophils [70]. *S. aureus* Wood 46, which lacks protein A, did not stimulate histamine release [71]. These results suggest that protein A mediates the *Staphylococcus*-induced activation of basophils. Skin and lung are involved in *S. aureus* infection [62–64]. It was therefore of interest to examine the effect of protein A on tissue mast cells. *S. aureus* Cowan 1 and soluble protein A effectively induced histamine release from both human lung and skin mast cells [72].

The classical protein A binding site for immunoglobulins binds the Fcγ of IgG1, IgG2, and IgG4 [60], whereas the alternative site binds the Fab portion of a percentage of IgG, IgM, IgA, and IgE [61]. Hyperiodination selectively alters the Fcγ-binding region of protein A [71]. The finding that the histamine-releasing activity of protein A was only slightly reduced by hyperiodination suggested that activation of FcεRI+ cells does not occur through the classical binding site of the protein [72]. Support for this hypothesis came from the effect on protein A-induced basophil activation of molecules that have only Fcγ-protein A reactivity, e.g. rabbit IgG, and those with both Fcγ and $F(ab')_2$ reactivity, e.g. human IgG. Whereas human IgG concentration-dependently inhibited protein A-induced histamine release, rabbit IgG, which does not bind the alternative site of protein A, did not [71, 72]. From this finding, one may conclude that protein A induces histamine release by binding to immunoglobulins bound to FcεRI+ cells through the alternative site.

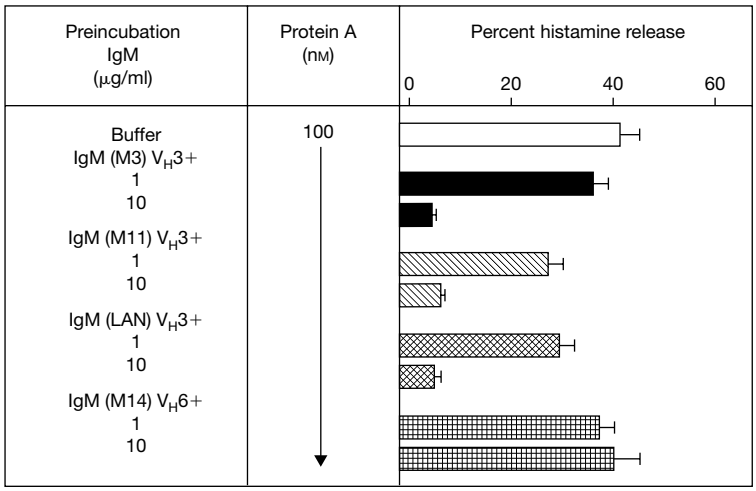

Fig. 4. Effect of preincubation of protein A with monoclonal IgM V_H3+ or IgM V_H6+ on histamine release from human basophils. Protein A (100 nM) was preincubated for 10 min at 37°C with increasing concentrations (1–10 μg/ml) of human monoclonal IgM V_H3+ (M3, M11, and LAN) or IgM V_H6+ (M14). Basophils were then added, and incubation was continued for another 30 min at 37°C. Each bar shows the mean ± SEM of triplicate determinations. (Reproduced with permission from Marone et al. [16].)

We carried out cross-desensitization experiments to examine the relationships between protein A and IgE. FcεRI+ cells preincubated with protein A under nonreleasing conditions and then challenged with anti-IgE released less than 5% histamine. In line with this finding, cells desensitized to protein A released approximately 90% less histamine when challenged with anti-IgE. This set of experiments indicates that the releasing activity of protein A is mediated mainly by interaction with IgE present on the membrane of FcεRI+ cells. The concept that protein A induces mediator release by binding to IgE is supported by the finding that protein A does not induce histamine release from FcεRI+ cells stripped of IgE [72].

Preincubation of protein A with human monoclonal IgM V_H3+ concentration-dependently inhibited histamine release from basophils (fig. 4). In contrast, a monoclonal IgM V_H6+ did not affect the histamine-releasing capacity of protein A. Based on these results, it appears that protein A activates basophils by interacting with IgE V_H3+.

Taken together, these findings are compatible with the hypothesis that the reactivity of protein A for human immunoglobulin is at least divalently expressed in the molecule [71, 72]. Thus, protein A can function as a bacterial superallergen that reproduces the releasing activity of anti-IgE and antigens on

FcεRI+ cells. These results support the hypothesis that protein A and other bacterial immunoglobulin superantigens can cause or exacerbate certain allergic disorders [62–69] through this mechanism that involves human FcεRI+ cell activation.

Protein L Activates Human FcεRI+ Cells through Interaction with κ Light Chains of IgE

Protein L is a cell wall protein of the anaerobic bacterium *P. magnus* [14]. It is constituted by up to five repeated immunoglobulin-binding domains (B_1–B_5) [73] and appears to be a virulence determinant [74]. Each homologous domain binds with high affinity to κ L chains without disrupting the function of the antigen-binding site [75, 76]. Protein L binds to the V domain of the $V_{\kappa I}$, $V_{\kappa III}$ and $V_{\kappa IV}$ subgroups but does not bind to immunoglobulin H chains, λ-chains, the C_L domains of κ L chains, or the V domain of the $V_{\kappa II}$ subgroup [77]. Thus, protein L binds human immunoglobulin irrespective of the H chain class, is mitogenic for B cells [78], and is an immunoglobulin superantigen.

We previously showed that protein L induces proinflammatory mediator release from human basophils and mast cells, probably by interacting with FcεRI-bound IgE [72, 79]. We next evaluated whether protein L and a fragment of protein L denoted 'B_1–B_4', which comprises four of the five immunoglobulin-binding repeats [73], induce cytokine (IL-4 and IL-13) synthesis and secretion from human basophils. Protein L and B_1–B_4 stimulated IL-4 release from basophils [80] and there was a significant correlation between IL-4 release induced by protein L and by B_1–B_4. These data demonstrate that protein L induces the secretion of IL-4 and IL-13, which are important for the polarization of Th2 cells [81], from basophils.

We also carried out a series of experiments to evaluate the mechanism of action of protein L on basophils. Brief exposure to a low pH that removes most of the IgE bound to basophils [18, 55] completely blocked the effect exerted by both protein L and B_1–B_4 on cytokine release from basophils. We further examined the relationship between protein L and anti-IgE using cross-desensitization between the two stimuli. The IL-4 release induced by protein L or anti-IgE was greatly reduced when cells had previously been challenged with anti-IgE or protein L, respectively. Basophils desensitized with protein L or anti-IgE remained responsive to anti-FcεRIα, which activates a specific epitope on the α-chain of FcεRI. Consequently, we may conclude that the releasing property of protein L is mediated by interaction with IgE present on basophils.

Protein L binds with high affinity to the L chains of human immunoglobulin [75]. Moreover, the multiple binding sites of protein L for κ L chains

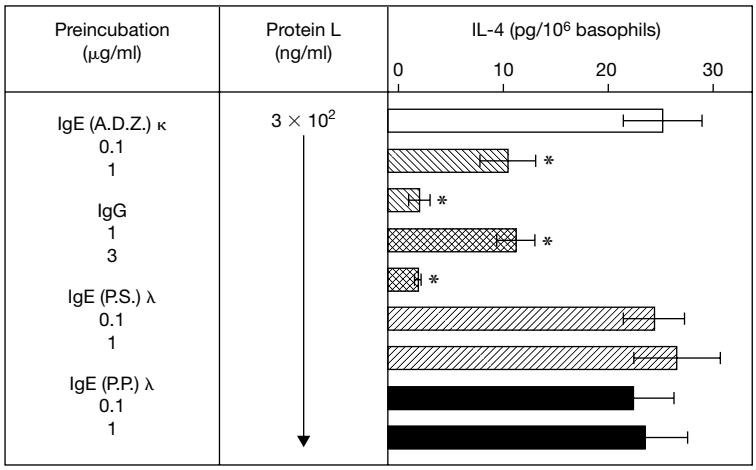

Fig. 5. Effects of preincubation of protein L with human monoclonal IgE (A.D.Z., P.S., P.P.) and human polyclonal IgG on IL-4 release from basophils. Protein L was preincubated for 15 min at 37°C with human monoclonal IgE (A.D.Z., P.S., P.P.), or polyclonal IgG. Basophils were then added, and incubation continued for another 4 h at 37°C. Each bar shows the mean ± SEM of IL-4 release obtained from three experiments. *$p < 0.01$ when compared with the group not preincubated with human immunoglobulin. (Reproduced with permission from Genovese et al. [80].)

[73, 75] render this protein similar to divalent anti-IgE antibodies [18]. As indicated above, protein L does not have affinity for λ L chains [75]. Preincubation of protein L with IgE myelomas from patients P.S. and P.P. displaying λ L chains did not affect the releasing activity of protein L, whereas IgE from patient A.D.Z. (κ-chains) and human polyclonal IgG (approx. 60% κ-chains) blocked the IL-4-releasing activity of protein L (fig. 5). These results indicate that human monoclonal IgE in A.D.Z. (κ) and polyclonal IgG competitively inhibit the interaction between protein L and IgE at the basophil surface. Our results also indicate that protein L from *P. magnus* and fragment B_1–B_4 are potent inducers of the synthesis and release of IL-4 and IL-13 from human FcεRI+ cells. The activity of protein L and B_1–B_4 is probably mediated by interaction with the κ L chains of IgE present on basophils. Protein L acts as a bacterial immunoglobulin superantigen by interacting with the κ L chains of IgE on human basophils to induce the synthesis and release of IL-4 and IL-13 [80].

In conclusion, our results demonstrate that different bacterial products (protein A and protein L) specifically activate human FcεRI+ cells to release

proinflammatory mediators and cytokines acting as immunoglobulin superantigens (superallergens).

Concluding Remarks

Mast cells and basophils are the primary effector cells of allergic reactions in humans. They can be activated in vitro by several IgE- and non-IgE-mediated immunological mechanisms, which are implicated in the pathogenesis of various subtypes of disorders. Although most IgE-mediated allergic reactions result from the interaction between multivalent allergens and specific IgE on the surface of human FcεRI+ cells, it is possible that autoantibodies against the IgE-FcεRI network are involved in certain allergic disorders. For example, anti-IgE autoantibodies or autoantibodies against epitopes of the α-chain of FcεRI have been identified in the plasma of some patients affected by chronic idiopathic urticaria [82]. Moreover, IgG anti-IgE autoantibodies from some patients with atopic dermatitis can induce the release of preformed and de novo synthesized mediators from human FcεRI+ cells [83].

It is well documented that certain viral infections are associated with the induction and/or exacerbation of allergic reactions [84–86]. Moreover, infection with HCV, HAV and HBV can be associated with increased IgE levels [30, 31] and certain allergic manifestations [28]. In addition, HIV-1 infection can be associated with increased IgE levels [33–42] and augmented prevalence of adverse reactions to drugs and urticarial rash [47–48]. Similarly, certain bacterial infections (e.g. *S. aureus*) can exacerbate atopic dermatitis [62–64], certain forms of allergic rhinitis [65, 66], and asthma [87]. Therefore, it appears that viral and bacterial infections can be involved in the induction and/or exacerbation of respiratory and skin allergies.

In these studies, we evaluated the possibility that endogenous proteins induced in vivo by viruses (e.g. protein Fv), viral proteins (e.g. gp120) or bacterial proteins (e.g. protein L and protein A) can immunologically activate human FcεRI+ cells to release proinflammatory mediators and cytokines.

Our results have highlighted a new mechanism by which endogenous, bacterial and viral proteins specifically activate FcεRI+ cells thus acting as immunoglobulin superallergens. Figure 6 schematically depicts a new pathogenetic link between viral and bacterial infections and tissue injury. The in vivo implications of IgE-mediated activation of human FcεRI+ cells by these inmunoglobulin superallergens have yet to be defined. It is conceivable that endogenous, bacterial or viral superallergens play a pathophysiological role in certain forms of allergic disorders.

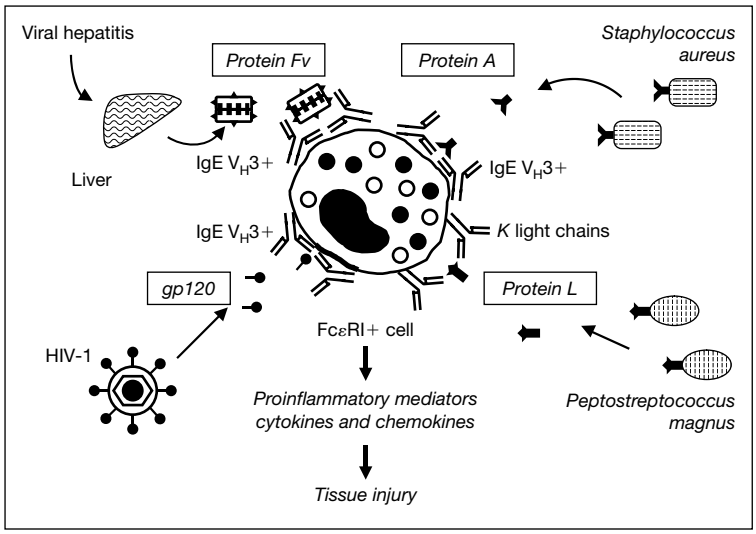

Fig. 6. Schematic representation of a novel mechanism by which endogenous (protein Fv), bacterial (protein A and protein L) and viral proteins (gp120) activate human FcεRI+ cells, acting as immunoglobulin superantigens. Protein Fv, synthesized in low amounts in normal liver, is released in the biological fluids of patients affected by acute and chronic viral hepatitis. Protein Fv has six binding sites for the V_H3 region of human immunoglobulin and is a potent stimulator of histamine and cytokine release from FcεRI+ cells through the interaction with V_H3+ IgE. HIV-1 gp120, a member of the immunoglobulin superantigen family, activates human FcεRI+ cells through interaction with membrane-bound IgE V_H3+. Protein A and protein L are two immunoglobulin superantigenic bacterial products that can activate human FcεRI+ cells. Protein A, a cell wall protein of *S. aureus*, has a classical binding site for Fcγ and an alternative site that binds the Fab portion of 15–50% of human polyclonal IgM, IgA, IgG, and IgE. Protein A induces mediator release from basophils and mast cells through the interaction with IgE V_H3+. *P. magnus* is a bacterium expressing a cell wall protein L that binds human immunoglobulin through high-affinity interaction with κ L chains, and is thus an immunoglobulin superantigen. Protein L activates human FcεRI+ cells through the interaction with the κ L chain of membrane-bound IgE.

Acknowledgements

This work was supported in part by grants from the *Istituto Superiore Sanità* (AIDS Project 40.C.60 and 40.D.57), *Ministero della Salute* (Alzheimer Project), *Ministero dell'Università e della Ricerca* (MUR) (Rome, Italy) and *Regione Campania* (Naples, Italy).

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Marone G (ed): Superantigens and Superallergens.
Chem Immunol Allergy. Basel, Karger, 2007, vol 93, pp 214–236

Role of Staphylococcal Superantigens in Airway Disease

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Abstract

Gram-positive *Staphylococcus aureus* germs constitutively have the possibility to release classical and egc-locus-derived enterotoxins which show superantigen activity, and effectively modify the functions of T and B cells, eosinophils, and other inflammatory and structural cells. The stimulation may lead to a type 2 T helper cell-polarized eosinophilic inflammation as well as a multiclonal IgE production, aggravating airway disease in the upper and lower respiratory tract. Recently, *S. aureus* has been demonstrated to reside intraepithelially, and potentially to release superantigens into the tissue from within the epithelial cells. An immune defect, either in the innate or adaptive immunity, might be responsible for this phenomenon. Follicle-like structures and lymphocyte accumulations, specifically binding enterotoxins, can be found within the mucosal tissue. Interestingly also, IgE antibodies to enterotoxins can be found in the majority of aspirin-sensitive patients, in nasal polyps and severe asthma alike. We here summarize the current evidence from animal and human studies for an active role of staphylococcal enterotoxins (SEs) in allergic rhinitis, nasal polyps, asthma, chronic obstructive pulmonary disease and finally early childhood wheezing, and discuss the similarities between those disease manifestations. As a principle, the occurrence of IgE antibodies to SEs correlates with disease severity in terms of total IgE formation, inflammatory markers and clinical expression of the disease. Preliminary evidence from animal models underlines the high potency of SEs to induce or modulate disease, and a few pilot intervention trials may serve as proof of concept for the impact of SEs on disease severity. However, therapeutic approaches are so far limited and empirical, and need further improvement to tackle this currently underestimated clinical challenge.

Gram-positive *Staphylococcus aureus* germs, to facilitate colonization and impair the host's self-defense, constitutively have the possibility to release a bunch of enterotoxins which show superantigen activity, and effectively modify

the functions of T and B cells, eosinophils, and other inflammatory and structural cells [1, 2]. The classical *S. aureus* enterotoxins comprise SE A–E and toxic shock syndrome toxin-1 (TSST-1). However, further enterotoxins have been described recently, derived from the egc gene locus [3]; these seem to be of relevance, as they frequently are produced by nasal *S. aureus*, and partially are unrelated to the production of classical enterotoxins [van Zele, unpubl. obs.]. Staphylococcal enterotoxins (SEs), as well as molecules derived from *Streptococcus pyogenes* [4] and some viruses [5, 6], are able to activate T cells via the T cell receptor (TCR)-major histocompatibility complex (MHC) class II complex, independent of the antigen-specific groove, by binding to the variable β-chain of the TCR. The susceptibility of a T cell to superantigens therefore is dependent on the usage of a specific β-chain repertoire, possibly leading to the activation of abundant T cells in a given tissue (normally, far less than 1% of T cells are activated by a specific antigen). Another recently described possibility of modifying the response to superantigens is based on the finding that HLA-DQ polymorphisms may alter the binding of superantigens to MHC class II [7]. Thus, the resulting response of a T cell population in a given tissue is dependent on many factors, such as production of and exposure to *S. aureus* enterotoxins, the intactness of the epithelial barrier, as well as the specific composition of TCRs and MHC class II on immune cells. Once activated, T cells would produce interleukins including IL-4, IL-5, IL-13, eotaxin and many others, which would lead to a severe eosinophilic inflammation and local IgE production. Other direct actions of superantigens on B cells, epithelial cells, or eosinophils have been described, which are summarized in a recent review [8] and other chapters of this book. All of these actions add to the enormous inflammatory potential of *S. aureus*-derived superantigens.

About 25% of the population is a permanent carrier of *S. aureus* in the nostrils, and approximately 20% of all human staphylococcal infections are autogenous [9]. The finding of IgE antibodies to SEA and SEB in nasal polyp (NP) tissue homogenates [10] indicated that these superantigens could be involved in the pathogenesis of nasal polyposis. NPs are characterized by abundant eosinophils, T cell activation, overproduction of IgE, and originally were thought to represent an allergic disease [11–14]. In western countries, more than 70% of polyps show tissue eosinophilia, and increased migration, activation and prolonged survival of eosinophils [12]. The concentrations of total IgE, IL-5, eotaxin, eosinophil cationic protein (ECP), cysteinyl leukotrienes (LTs), and the soluble low-affinity IgE receptor (CD23) are significantly higher in polyp tissue compared to controls. Total IgE is significantly correlated to IL-5, ECP, $LTC_4/D_4/E_4$ and sCD23, and to the number of eosinophils. Recent evidence accumulates, however, that *S. aureus* enterotoxins, acting as superantigens, induce a substantial inflammatory reaction in a large subgroup of NPs, and

strongly modify the disease [10]. An important subgroup of those polyp patients demonstrated a multiclonal IgE formation, including IgE to *S. aureus* enterotoxins, a high total IgE level, increased levels of ECP and IL-5, and a high prevalence of asthma. These studies suggested that bacterial superantigens could induce IgE synthesis in NPs and impact the degree of eosinophilic inflammation [10, 15].

There is recent evidence that *S. aureus* enterotoxins also affect the airways, and modify diseases such as allergic rhinitis, nasal polyposis, severe late-onset asthma, chronic obstructive pulmonary disease (COPD) and early childhood persistent wheeze. We here summarize the current evidence for an active, at least modifying role of superantigens in upper and lower airway disease, and speculate on the possible clinical implications for the management of patients affected.

S. aureus Colonization and Immune Response to Staphylococcal Enterotoxins Is Increased in Allergic Rhinitis

Factors that modify the expression of rhinitis have been reported to be environmental factors (temperature, smoking, and air pollution), sex hormones, stress and alcohol. Although for other disorders including bronchial asthma and atopic dermatitis, it has been reported that bacteria could play a role in the pathogenesis of allergic inflammatory diseases, there are few reports regarding the association between bacterial colonization and allergic rhinitis. *S. aureus* is a representative constituent of bacterial flora in the nasal cavity and a common pathogen producing several enterotoxins with superantigenic properties, which could modify inflammatory reactions such as allergic rhinitis.

To gain insight into a possible bacterial impact on allergic rhinitis, Shiomori et al. [16] examined the nasal carriage of *S. aureus* and its superantigen production. The rate of the nasal carriage of *S. aureus* was significantly higher in patients with perennial allergic rhinitis (44%) than that of control subjects (20%, $p < 0.01$). Moreover, the rate of the nasal carriage of superantigen-producing *S. aureus* in the patients (22%) was significantly higher than that of the control subjects (6.7%, $p < 0.5$). The nasal symptom scores of the *S. aureus*-positive patients were significantly higher than those of the *S. aureus*-negative patients ($p < 0.05$), although there was no significant association between symptom scores and superantigen production. The reason for the higher carriage rates in patients with perennial allergic rhinitis has remained unclear so far.

Rossi et al. [17] determined the prevalence of serum-specific IgE towards SEA, SEB, SEC, SED and TSST-1 in patients suffering from persistent allergic

rhinitis and/or allergic asthma and confirmed an increase in serum ECP in patients with IgE antibodies to SEs. Furthermore, these patients showed significantly increased concentrations of total IgE and house dust mite-specific IgE antibodies in serum compared to IgE-negative allergic rhinitis patients.

In another study, *S. aureus* was found significantly more often in nasal lavages of house dust mite-allergic patients versus controls [18]. Comparing allergic *S. aureus* carriers with allergic noncarriers, nasal symptom scores tended to be higher, and the eosinophilic cell activation marker ECP and nasal total IgE levels were significantly higher in the carriers. Nasal *S. aureus* carriers had a higher nasal IL-13/IFN-γ ratio, and this was correlated with higher nasal total IgE in allergic patients. From these data, it can be concluded that nasal *S. aureus* carriage is not only a frequent phenomenon in patients with persistent allergic rhinitis, but also actively modulates the disease by promoting local IgE production and eosinophilic inflammation.

These observations in humans could recently be confirmed by animal experiments investigating the in vivo effect of nasal exposure to SEs on the development of allergic rhinitis [19]. BALB/c mice were intranasally sensitized with *Schistosoma mansoni* egg antigen (SmEA) in the presence or absence of SEB. Control mice were sensitized with either SEB or SmEA alone. Nasal exposure to SEB enhanced the development of allergic rhinitis in SmEA-sensitized mice, as manifested by SmEA-specific IgE production, nasal eosinophilia, and IL-4 and IL-5 production by nasal mononuclear cells after antigen challenge. In addition, these mice produced SEB-specific IgE whereas mice treated with SEB without SmEA sensitization did not produce SEB-specific IgE nor demonstrate nasal eosinophilia. These results suggest that the nasal exposure to SEB enhances susceptibility to and modifies allergic rhinitis although the exposure to SEB solely does not induce allergic rhinitis.

Enhanced Immune Response to *S. aureus* in Nasal Polyposis

The vast majority of bilateral NPs are associated with a prominent eosinophilic inflammation and elevated local IgE levels in polyp fluid [10, 20]. In a retrospective study by Settipane and Chafee [21], polyps were present in 2.8% of atopic patients, but in 5.2% of nonatopic subjects. Furthermore, NPs occur more frequently in subgroups of patients with asthma and aspirin sensitivity [21]. About 40–80% of patients with aspirin sensitivity suffer from polyposis, and about 15% of polyp patients are hypersensitive to aspirin [21]. In studies involving large series of patients with nasal polyposis, asthma was found in 20–70%, and nonallergic asthma is significantly more frequently linked to polyps compared to allergic asthma [21].

Characterization of NPs suggests a central deposition of plasma proteins (albumin), regulated by the subepithelial, mainly eosinophilic inflammation, as pathogenetic principle of polyp formation and growth. The accumulation and activation of eosinophils is favored by the low concentrations of TGF-β_1 and overproduction of IL-5 and eotaxin in NP tissue [20]. Although elevated IgE levels are found in NPs, total IgE and IgE antibodies in NP tissue were unrelated to skin prick tests, but correlated with the degree of eosinophilia. In about half of the polyp specimens, we demonstrated polyclonal IgE formation, specific IgE to *S. aureus* enterotoxins, high levels of local IgE antibodies, and a high prevalence of asthma [10]. Similarly, we demonstrated that IgE antibodies to SEs were more often found in serum from patients with severe asthma than in those with mild asthma [22].

S. aureus frequently colonizes the nostrils in healthy subjects, and can be found in acute and chronic rhinosinusitis (CRS) [16]. We have recently reported an increased colonization rate of *S. aureus* in NPs, but not in CRS without polyps [23]. Colonization with *S. aureus* was present in 63.6% of subjects with polyps, with rates as high as 66.7 and 87.5% in the subgroups with asthma and aspirin sensitivity, respectively, which were significantly higher than in controls and subjects with CRS (33.3 and 27.3%, respectively). Furthermore, repeated swabbing of the middle meatus in 8 subjects with polyps suggested long-term colonization with *S. aureus*. IgE antibodies to *S. aureus* enterotoxins, using a combination of different enterotoxins in a screening assay, were present in 27.8% in polyp samples, with rates as high as 53.8 and 80% in the subgroups with asthma and aspirin sensitivity, respectively, compared to 15% in controls and 6% in subjects with CRS [23]. The concentration of ECP, reflecting the eosinophilic inflammation, was significantly increased in polyp samples with the presence of IgE antibodies to enterotoxins versus samples without IgE, confirming a strong inflammatory effect of superantigens. In subjects with NPs and comorbid asthma or aspirin sensitivity, rates of colonization and IgE response in nasal tissue homogenates were further increased, paralleled by increases in ECP and total IgE. These figures indicate that there is a strong relation between staphylococcal colonization and tissue immune responses to enterotoxins in NPs, which may even be reflected in lower airway comorbidity (table 1) [23].

Comparable rates of colonization with *S. aureus* (71%) and IgE antibody formation (50%) to superantigens were found in another polyp study, with low rates in control subjects (25 and 0%, respectively), confirming our first results [18]. Colonization rates always exceeded those of IgE immune response to *S. aureus* enterotoxins, indicating that colonization may not necessarily lead to the production or contact of superantigens with the immune system. Until now, *S. aureus* has been regarded as a noninvasive extracellular pathogen [19]. However, recent findings demonstrate the ability of this germ to invade nonphagocyting

Table 1. Mediators of IgE in control turbinate mucosa and nasal polyp tissues with and without IgE to *S. aureus* enterotoxins (SAE(+) and SAE(−)). Inflammatory mediators are up-regulated in SAE(−) polyps vs. controls, and further amplified in SAE(+) vs. SAE(−) polyps (significant data in bold, significance between all groups (*)). In SAE(+) polyps, changes are also reflected in serum (significancee vs. controls and vs. SAE(−)NP (**))

	Controls	NP-SAEs(−)	NP-SAEs(+)	
Tissue				
ECP (μg/ml)	602.5 (IQR: 309.9–894.3)	**9,806.9** (IQR: 1,686.5–17,673.8)	**25,583.0** (IQR: 17,226.0–29,870.3)	$p < 0.0001$ (*)
IL-5 (pg/ml)	20.9 (IQR: 16.9–25.0)	**81.5** (IQR: 38.9–291.9)	**327.9** (IQR: 106.2–385.5)	$p < 0.0005$ (*)
MPO (ng/ml)	4,882.8 (IQR: 3,007.1–7,015.0)	8,013.9 (IQR: 4,912.1–11,476.0)	9,705.2 (IQR: 7,426.1–17,427.1)	
Total IgE (kU/l)	1.93 (IQR: 1.9–1.9)	**323.9** (IQR: 67.2–387.5)	**1,564.0** (IQR: 739.1–2,039.7)	$p < 0.0005$ (*)
Specific IgE to SAEs (kU_A/l)	BDL	BDL	**8.6** (IQR: 6.3–17.0)	$p < 0.0005$ (*)
Serum				
ECP (μg/ml)	9.0 (IQR: 3.8–14.1)	10.9 (IQR: 7.1–32.7)	**22.4** (IQR: 16.4–36.9)	$p = 0.0467$ (**)
MPO (ng/ml)	11.8 (IQR: 8.3–13.1)	7.5 (IQR: 3.7–16.7)	10.3 (IQR: 5.9–13.4)	
Total IgE (kU/l)	21.8 (IQR: 8.9–56.0)	37.2 (IQR: 20.8–215.2)	**211.2** (IQR: 152.5–431.5)	$p = 0.0064$ (**)
Specific IgE to SAEs (kU_A/l)	BDL	BDL	**0.4** (IQR: 0.1–1.3)	

eukaryotic cells, and to possibly persist there for weeks. *S. aureus* small-colony variants are a naturally occurring slowly growing subpopulation which has recently been related to chronic recurrent antibiotic-resistant infections such as cystic fibrosis [24]. It has been demonstrated that *S. aureus* invades cultured cells of nonprofessional phagocytes and cell lines [25], as well as human respiratory epithelial cells [26]. Analysis of invaded cultured cells by electron microscopy revealed *S. aureus* in vacuoles within the airway epithelium [27]. The interaction between *S. aureus* and epithelial cells has been proposed to occur through binding of fibronectin-binding proteins on germs to fibronectin, β_1-integrins and heat shock protein 60 [28, 29]. The ability to be internalized and survive within host cells may explain the refraction of polyp disease to antibiotic

treatment, which represents a hallmark of polyposis, as well as the chronicity of disease and recurrence, months and even years after apparently successful therapy. Antibiotics commonly used for the management of *S. aureus* infections appear to create a niche for invasive intracellular *S. aureus* [30].

We have recently used immunohistochemistry to demonstrate the presence of *S. aureus* and the production of SEB in samples from polyps. Intraepithelial staining for *S. aureus* was found in a substantial subgroup of polyps, with affected and unaffected areas coexisting in the same samples [Patou, unpubl. obs.]. SEB could be co-localized to the intracellular Staphylococcus, indicating the potential of releasing this enterotoxin into the tissue. Apoptotic epithelial cells with their contents, and *S. aureus* which crosses the basal membrane, would be taken up by macrophages, which have been shown to be prevalent in increased numbers in NPs versus controls [31]. These macrophages in NP tissue have been characterized as CD68+, macrophage mannose receptor-positive, CD163+, and RFD7+ phagocyting macrophages, which characterize a mature phenotype of macrophages. Surprisingly, there was a significant lack of staining for *S. aureus* in macrophages in the lamina propria in polyp tissues compared to controls. These new data suggest a reduced capacity of these macrophages to phagocyte *S. aureus*, which needs further functional investigation. Of interest, the lack of defense against *S. aureus* seems to give rise to a local immune response to SEs, as measured by increased IgE antibodies to enterotoxins, total IgE, ECP and IL-5 versus controls in this patient group. In the skin, another possible deficiency in innate immunity, namely the lack of defensins, has been proposed [32], which we could not confirm in our studies on NPs [33]. Furthermore, a deficit in IgG2 antibodies against SEC_1 has been described recently, the clinical relevance of which is currently unclear [34] and has to be studied also in polyps. However, if confirmed, this finding could indicate a deficiency not only in the innate, but also in the adaptive immune regulation, which could predispose to develop staphylococcal superantigen-driven disease.

When NPs were analyzed for T and B lymphocytes and IgE by immunohistochemistry, follicular structures were found in 25% of the samples, and diffuse lymphoid accumulations were seen in all NP samples [35]. Follicle-like structures are composed of T and B lymphocytes, and stain positive for IgE and the low-affinity IgE receptor, whereas the high-affinity receptor is found outside the follicle only. Plasma cells expressing CD38 are prominent in the lymphoid accumulations, which also stain positive for IgE, CD3, FcεRI, but not for CD23 [35]. These lymphocyte accumulations therefore may be considered to develop from follicle-like structures, with B cells maturing into IgE-producing plasma cells. Interestingly, we demonstrated binding of biotinylated SEA to follicular structures and lymphoid accumulations in polyp tissue. The specificity

of the SE binding was confirmed by staining with an excess of nonbiotinylated SEA to biotinylated SEA, which completely blocked the signal. Furthermore, no follicular structures or SE staining were found in control tissue. These data suggest an organization of secondary lymphoid tissue with polyclonal B cell activation in NPs due to chronic microbial colonization and stimulation by enterotoxins, which is likely to be the cause of IgE switch and formation.

There is increasing evidence that SEs can directly affect the frequency and activation of the B cell repertoire. Functional studies in B cells have shown that *S. aureus* protein A induces proliferation of these cells [36]. Studies with TSST-1 indicated that staphylococcal superantigens may play an important role in the modulation of allergic disease, since they may augment isotype switching and synthesis of IgE, both in vitro [36] and in vivo, in an SCID mouse model [37]. Although TSST-1-induced activation of B cells in vitro is indirect and dependent on increased expression of CD40 ligand on T cells, a more recent study has provided evidence for also a direct effect by demonstrating TSST-1-induced expression on B cells of B7.2 [38], a molecule that has been shown to enhance type 2 T helper cell (Th2) responses and to be involved in IgE regulation. In mucosal tissues of hay fever and asthma patients, mRNA for the ε-chain of IgE was found in a significant proportion of B cells using in situ hybridization [39–41], supporting the hypothesis of a truly local IgE synthesis in the airway mucosa. Pilot studies on the expression of costimulatory signals such as CD40/CD40 ligand and CD28/B7 in lymphocytes of NPs support this notion [van Zele, unpubl. data], and studies on local IgE switching events are currently performed [42]. However, there is indirect evidence of a local production of IgE: total IgE and IgE antibody concentrations to enterotoxins were in all cases higher in tissue compared to serum, but SE-specific IgE antibodies may be detected in the serum of polyp patients [15], especially when asthma coexists. The IgE/albumin ratios in polyp tissue and in serum were dissociated, again indicating that tissue IgE is rather the result of a local IgE production than of extravasation. Furthermore, IgE antibodies in polyp tissue only showed a partial relation to IgE antibodies in serum and to skin prick test results. In a substantial subgroup of patients, the typical pattern of IgE expression in polyp tissue was found: a polyclonal type of IgE expression with IgE antibodies to common aeroallergens and a high level of total IgE. These findings resemble those in atopic dermatitis, where colonization of the inflamed skin with *S. aureus* clearly contributes to the high IgE levels in serum and to the severity of the disease [43].

Detailed analysis of IgE expression in serum and tissue of NP patients reveals two patterns: ‘the allergic type’ and ‘the polyclonal type’ that can be found either isolated or combined [35]. The ‘allergic’ type of IgE expression is characterized by increased concentrations of total IgE and IgE antibody specificities in nasal

tissue that correspond to those in serum and to the skin prick test results. In contrast, the 'polyclonal' IgE expression is a local process and IgE antibodies found in polyp tissue are only partially reflected in serum of the same patients and are independent of the skin prick results. Notably, this polyclonal expression is associated with hyperimmunoglobulinemia and only a small fraction of the total can be explained by IgE antibodies. Polyclonal expression was described in 16/24 NP tissues and was associated with IgE antibodies to SEs in 12 cases, indicating that other than the classical enterotoxins might have acted as superantigens [35]. Additionally, we found a higher incidence of *S. aureus* colonization (17/24) and IgE antibodies to SEs in NP tissue (12/24) compared to controls (3/12 and 0/12, respectively). An augmented local synthesis of IgE may, under appropriate circumstances, increase allergic reactivity, but when it is excessive, it could suppress specific reactivity by saturation of Fcε receptors on mast cells through polyclonal IgE and/or an inhibition of specific antibody synthesis to environmental allergens by the polyclonal response. These mechanisms could explain why nasal symptoms and markers of nasal mucosal inflammation did not increase in relation to natural seasonal allergen exposure in highly ragweed-allergic patients with polyps and why nasal provocation in NPs is largely unsuccessful although elevated IgE levels are present. However, the backside of this local polyclonal IgE production may be the permanent triggering of the IgE-mast-cell-FcεRI cascade that maintains polyp growth.

In a recent study investigating the production of IgE, IgG, IgM, IgA and IgG subclasses in tissue homogenates and serum from patients with NPs, CRS and control patients, we found IgA, IgG and IgE concentrations to be significantly higher in tissue homogenates, but not in the serum of NP compared to CRS patients and controls. In line with these findings, naïve CD19+ B cell and plasma cell counts (CD138+) were significantly higher in NP tissue. Furthermore, within the NP group, those with specific IgE to SEs had significantly higher concentrations of IgG and IgE, and also showed a significantly higher fraction of IgG4 and a lower fraction of IgG2 than those without specific IgE production [van Zele, unpubl. data]. This observation indicates the usage of IgG4 for the immunoglobulin switch to IgE; since NPs have a Th2-polarized inflammation with extremely high concentrations of IL-5, the presence of small amounts of IL-4 most likely diverts the immunoglobulin production towards IgE synthesis in NP tissue.

Previous studies by Coker et al. [41] showed evidence that a local clonal expansion, somatic hypermutation and class switching occurs in the nasal mucosa. More specifically, they found a biased use in the repertoire of IgE heavy-chain variable regions (V_H) [44]. The significant bias in tissue towards V_H5 suggests that a local event, possibly superantigens, may cause the V_H5 bias. Such a V_H5 bias was also found in other diseases, especially in atopic dermatitis, a disease mediated by *S. aureus* and its enterotoxins.

The local hyperimmunoglobulinemia in NPs points towards an extreme immunological defense mechanism during which a polyclonal immunoglobulin production is maintained [35]. Under normal conditions, contact with antigen leads to a short-term serological memory which is driven by short-lived and long-lived plasma cells. This antigen-driven proliferation and differentiation of memory B cells to short-lived plasma cells induces high levels of protective antibodies [45]. However, there also exists a long-term serological memory which is mediated by an antigen-independent polyclonal activation of memory B cells leading to a sustained production of plasma cells of all memory specificities [45]. Why our adaptive immune system produces these immunoglobulins in a polyclonal way remains fairly understood; it may serve as a protection against powerful bacterial toxins. Since there is no time to mount a secondary immunoglobulin response to achieve protection, the persistent and polyclonal immunoglobulin production relies entirely on preexisting and neutralizing antibodies [46].

Is There a Link between Staphylococcal Enterotoxins and Aspirin Sensitivity?

From the first study in patients with local IgE against SEs [10] it appeared that the highest IgE concentrations were obtained from samples of aspirin-sensitive subjects. We therefore extended our observations in a nonallergic, but severely inflamed subgroup of patients, who also suffered from asthma. Subjects with nasal polyposis from Poland were classified as aspirin-sensitive (ASNP) or aspirin-tolerant (ATNP) asthmatics, based on a bronchial aspirin challenge test [47]. Homogenates prepared from NP tissue were analyzed for concentrations of eosinophilic markers, total IgE and IgE antibodies to enterotoxins (SEA, SEC, TSST-1) [22], and compared to inferior nasal turbinates from healthy subjects.

As expected, a significant increase in IL-5 concentrations, total IgE and IgE antibodies to SEs was observed in supernatants from NP patients compared to controls, with concentrations of IgE to SEs correlating to IL-5 and ECP concentrations. NP patients were further analyzed in two groups, with or without aspirin sensitivity (ASNP and ATNP, respectively). Concentrations of total IgE and IgE antibodies to the mix of SEs were significantly higher in ASNP patients compared to the ATNP and control groups. Also, quantities of IL-5 and ECP were upregulated in ASNP patients and differed significantly from ATNP and control subjects. These results confirmed that the immune response to SEs was accompanied by the upregulation of eosinophilic inflammation markers in NPs, and suggested a possible link of SEs to aspirin

sensitivity, which might be direct (SEs inducing superantigen effect) or indirect (linked to the severity of inflammation). To further analyze this phenomenon, ASNP and ATNP patients were each divided into two subgroups, with and without IgE antibodies to SEs. Out of 13 patients with ASNP, 7 were SE+ in comparison to 7 out of 27 in the ATNP group and none out of 12 subjects in the control group. Concentrations of inflammatory markers (IL-5 and ECP) did not differ between ASNP SE+ and SE− groups, but were upregulated with respect to the control group. These results rather suggested an indirect relation between SEs and aspirin sensitivity, with the severity of inflammation as a link between both observations.

Our findings have recently been confirmed by Suh et al. [48], who also studied IgE antibodies to SEs and eosinophilic markers in Korean aspirin-sensitive and aspirin-tolerant asthmatics with NPs. These authors also found an increase in ECP, but not IL-5, between these groups, and significantly increased levels of IgE to SEs in aspirin-sensitive subjects. The authors confirmed the impact of SEs on NP disease in Korea, expanding on our European observations, and discussed a direct impact of SEs on the manifestation of aspirin sensitivity.

Further investigations, relating eicosanoid production and eosinophilic markers in CRS patients with and without NPs, and in NP patients with aspirin sensitivity, to data in normal nasal mucosa from healthy subjects, showed that LTC_4 synthase, 5-lipoxygenase and cysteinyl LT ($LTC_4/D_4/E_4$) concentrations increased with disease severity (per patient group) [49]. Other metabolites such as cyclooxygenase-2 and prostaglandin E_2 significantly decreased with disease severity. IL-5 and ECP were increased in both groups of NP tissues compared to controls and CRS, and correlated directly with $LTC_4/D_4/E_4$ and inversely with prostaglandin E_2 concentrations. These data confirmed the notion that changes of tissue eicosanoid metabolism do occur in CRS even in the absence of clinical aspirin sensitivity and appear to be related to the severity of eosinophilic inflammation.

More recently, we extended our observations demonstrating that the production of cysteinyl LTs, LTB_4, and lipoxin A_4 is upregulated in NP tissue of patients with an immune response to *S. aureus* enterotoxins versus SE-IgE-negative NP patients [50]. Again, the levels of the eicosanoids correlated with markers of eosinophil activation and survival (ECP and IL-5) and with concentrations of IgE antibodies to SEs. Taken together with former study results, we could confirm the amplifying role of *S. aureus* enterotoxins in upper airway inflammation, paving the way for the most severe form of sinus disease, namely nasal polyposis in aspirin-sensitive patients. So far, however, there is no evidence that the clinical manifestation of aspirin sensitivity is directly caused by SEs.

Staphylococcal Enterotoxins Provide a Link to Lower Airway Disease: Animal Experiments

Until recently, there has only been indirect evidence that SEs could possibly also impact lower airway disease, especially in poorly controlled asthma. By studying the TCR Vβ repertoire of bronchoalveolar lavage (BAL) cells and peripheral blood mononuclear cells (PBMCs) from subjects with poorly controlled asthma [forced expiratory volume in 1 s (FEV_1) <75%], subjects with well-controlled asthma and control subjects, Leung and coworkers [51] found a significantly higher expression of Vβ8+ T cells in BAL fluid of poorly controlled asthmatics compared to the other groups. Increased Vβ8+ BAL T cells were present in the CD4+ and CD8+ subsets, suggesting activation by SEs.

Experiments in mice to delineate the type of immune response triggered by superantigen exposure to the airway mucosa showed that a low dose of SEB could trigger an inflammatory response characterized by mucosal and airway recruitment of lymphocytes, eosinophils and neutrophils. These responses were associated with the development of increased airway responsiveness in SEB-treated mice, observed in IgE high responder BALB/c as well as in IgE low/intermediate responder C57BL/6 mice. These results suggested that the local immune response following mucosal superantigen administration triggers a unique inflammatory response in the airways in mice, resembling many features of 'intrinsic asthma' [52].

We took advantage of a mouse model of allergic asthma to study the effects of nasal or bronchial applications of SEB on the development of allergic asthma in previously sensitized mice [53]. Male BALB/c mice were kept under conventional pathogen-free conditions and were actively sensitized by 7 intraperitoneal injections of 10 μg ovalbumin (OVA) on alternate days from days 1 till 13 as described earlier [54]. Mice were then exposed daily for 5 min to nebulized OVA (OVA mice) or saline (SAL mice) from days 33 till 37. This protocol resulted in OVA-challenged mice in the induction of bronchial eosinophilia, Th2 cytokine production, bronchial hyperresponsiveness and elevated OVA-specific IgE titers in serum as previously published [55], whereas SAL mice did not show any bronchial inflammation. One hour prior to the latter airway challenge on days 33, 35 and 37, the nose and bronchi were exposed to 10 μl of saline with or without SEB at 500 ng.

In SAL mice, nasal application of SEB resulted in a significant increase in the total cell counts in BAL fluid. Compared to nasal application of SEB, endobronchial application of SEB induced a stronger bronchial inflammatory response in SAL mice. Similar observations were made when evaluating bronchial inflammation on histologic sections and by measuring the mean thickness of peribronchial inflammation.

Repeated exposure of sensitized mice to nebulized OVA (OVA mice) without SEB contact induced bronchial inflammation characterized by mainly eosinophils in the BAL fluid and influx of inflammatory cells in bronchial tissue, as illustrated on hematoxylin-and-eosin-stained sections. When SEB was administered onto the nasal mucosa during the development of bronchial allergic inflammation in response to OVA inhalation, bronchial inflammation was clearly aggravated. Compared to nasal application of SEB in OVA mice, bronchial administration of SEB in OVA mice equally aggravated the inflammatory response in the lower airways. Thus, in bronchial tissue of OVA mice, the inflammatory response was aggravated by SEB contact via the nose and the bronchi.

Compared to SAL mice that were exposed to saline in nose and bronchi, OVA mice presented with higher expression of mRNA for IL-5, eotaxin-1 and IFN-γ, whereas the expression of mRNA for IL-12 p40 was lower. In OVA mice, nasal application of SEB further elevated the expression levels of mRNA for IL-5, IL-4, eotaxin-1, IL-12 p40, IFN-γ and TGF-β. Bronchial application of SEB upregulated the expression of IL-4, IL-12 p40, IFN-γ and TGF-β, without significant effects on IL-5 and eotaxin. In addition to higher levels of mRNA for Th2 cytokines and IFN-γ in OVA mice, nasal application of SEB in OVA mice enhanced serum concentrations of IL-4, IFN-γ and to a lesser extent IL-5. Also, in SAL mice, nasal mucosal contact with SEB enhanced the systemic levels of IL-4 and IFN-γ in a significant way. Remarkably, bronchial application of SEB failed to significantly enhance the systemic levels of IL-5, IL-4 and IFN-γ, both in SAL as well as in OVA mice.

On day 41, measurement of total and OVA-specific IgE levels in the serum of SAL mice revealed that endonasal application of SEB increased IgE titers in a nonsignificant way, whereas endobronchial application of SEB stimulated the production of OVA-specific IgE and total IgE, resulting in a twofold increase in OVA-specific and total IgE titer in the serum. Compared to SAL mice, OVA mice present with higher levels of OVA-specific and total IgE titers, due to the boosting effect on IgE production by repeated exposures of the airways to OVA. When SEB was applied onto the nasal or bronchial mucosa of OVA mice, this did not affect IgE titers in the blood.

Thus, we demonstrated here in vivo that mucosal contact with SEB is capable of aggravating several features of bronchial allergic inflammation: bronchial eosinophilic inflammation, IL-4, IL-5 and IFN-γ levels in bronchi and systemic circulation, bronchial expression of eotaxin-1 and TGF-β, and allergen-specific IgE titers [53]. Not only bronchial but also nasal mucosal contact with SEB is capable of inducing a more severe bronchial allergic inflammation in sensitized mice.

It was also remarkable to observe that nasal applications of SEB in sensitized mice induced a similar degree of bronchial eosinophilia as was found after repeated inhalations of allergens by sensitized mice. This observation may shed new light on the pathophysiology of bronchial eosinophilia in asthma. Beside allergen inhalation and IgE-mediated mast cell degranulation and activation of Th2 cells, airway mucosal contact with SEB may give rise to an inflammatory response that shares many inflammatory characteristics with the allergen-driven pathway. The observation of nasal SEB being an aggravating factor for asthma fits in the concept of united airway disease. Nasal colonization with *S. aureus* may therefore play a role in the pathophysiology of bronchial symptoms in asthma patients.

S. aureus Enterotoxins Provide a Link to Lower Airway Disease: Human Evidence in Adults

Also in humans, evidence for a direct impact of enterotoxins on lower airway disease is growing. Based on our previous findings, we used a sensitive and highly specific screening tool, the SE mix, to detect IgE to SEs in serum of mild and severe asthmatics, classified by lung function and need for drug treatment, versus controls. IgE antibodies to the SE mix were found significantly more frequently in severe asthmatics (62%) versus controls (13%, $p = 0.01$), and were linked to concentrations of IgE antibodies in serum, severity of eosinophilic inflammation (ECP in serum), and corticosteroid dependence [22]. Thirty-one out of the 55 asthma patients showed increased concentrations of total IgE in serum (>100 kU/l), and 21 of those had IgE antibodies to the SE mix. Consequently, 10 subjects had an increased total IgE, but no IgE antibodies to the SE mix. Twelve sera had a total IgE above 500 kU/l, and 9 were positive, 3 negative for IgE antibodies to SEs. These data suggest that in some patients, other superantigens than the ones tested here may also play a role, e.g. Streptococcus. We therefore proposed a crucial role for SEs in the pathophysiology of upper and lower airway disease, linked to the severity of eosinophilic inflammation, total IgE synthesis, but also clinical disease severity, to be confirmed in larger population as well as in confirmatory treatment studies.

This study has now been extended, investigating asthmatics from mild disease (step 1 management) to chronic persistent disease requiring management at steps 4 or 5 of the British Thoracic Society (BTS) and the Scottish Intercollegiate Guidelines Network guidelines [56]. FEV_1 progressively declined from BTS step 1 (95.0 ± 9.3) to BTS step 2/3 (90.1 ± 12.7) and BTS step 4/5 (59.8 ± 19.9), and 41% of the severe asthmatics had required a hospital admission, 96% had required oral steroids within the last year. Levels of specific IgE

to SEs were significantly higher in the mild and severe asthmatics than in the controls, and positive results were found significantly more often in serum from severe asthmatics than mild asthmatics and controls (53.8 vs. 34.5 vs. 16.3%). These findings contrasted with the results of specific IgE measures against indoor aeroallergens, with over 70% of mild and moderate asthmatics having specific IgE against these common aeroallergens. However, there was no difference between the control subjects and severe asthmatics in the proportion of subjects demonstrating aeroallergen sensitization. Amongst all asthmatic groups, there was a significant correlation between levels of total IgE and levels of IgE to the SE mix.

Within each group, when subgroups defined by the IgE to the SE mix status were compared, total IgE was significantly higher in the SE+ subgroup. Aspirin-sensitive severe asthmatics were significantly more likely than aspirin-tolerant patients to have IgE to SEs (78.6 vs. 44.7%, $p < 0.05$). Severe patients with a history of NPs were also more likely to have IgE to SEs than those without a history of NPs (81.8 vs. 46.3%, $p < 0.05$).

These results clearly show the relationship between elevated levels of serum IgE to SEs and asthma severity. Such a relationship was not evident with respect to specific IgE against the common aeroallergens Der p1 or Fel d1. Indeed the percentage of subjects in the severe asthma group with common aeroallergen sensitivity was no different from that in the healthy control population, consistent with the higher proportion of classically nonatopic asthmatics that have severe disease. The findings identify that a high proportion of the severe asthmatics are not atopic in the common sense, but that the relevant allergens are bacterial enterotoxins rather than environmental aeroallergens. As indicated, histopathologically the airway mucosal features in classical, non-steroid-treated, nonatopic asthma are similar to those in atopic asthma, with cytokine expression compatible with a Th2 profile and airway eosinophil recruitment. The increased presence of specific IgE against SEs, indicating contact of the superantigens with the immune system, now provides an explanation for this.

We also studied the expression of total IgE and IgE antibodies to SEs in COPD patients, smokers without COPD, and healthy controls [57]. IgE antibodies to SEs were found in 1/10 controls and 1/16 smokers, but in 7/18 patients with stable disease (38.9%) and 21/54 patients with exacerbated COPD (38.9%). The IgE concentrations of patients with stable or exacerbated COPD were significantly higher than those of smokers or controls. Furthermore, IgE to SEs decreased significantly in the exacerbated patients during hospitalization, going along with a significant increase in FEV_1. These data suggest a role for superantigens in exacerbated COPD similarly to that in severe asthma.

Impact of *S. aureus* Sensitization on Allergic Diseases in Early Childhood

Based on in vitro data on the preferential release of Th2 cytokines from SEB-stimulated PBMCs, it was suggested that SEs could play an important role in the development of allergic diseases early in life, possibly representing a counterplayer to endotoxins released from Gram-negative bacteria which seem to prevent allergic diseases [58]. However, there is very little information on the association between SEs and allergic diseases in early childhood, as epidemiological data are lacking so far. Within the context of a population-based birth cohort study (Manchester Asthma and Allergy Study), A. Custovic, in cooperation with us, aimed to investigate the potential role of SEs in atopic disease during early childhood using SE-specific IgE as a marker [59]. The Manchester Asthma and Allergy Study is an unselected population-based birth cohort study described in detail elsewhere [60, 61]. Subjects were recruited from the antenatal clinics within the first trimester of pregnancy when all pregnant women were screened for eligibility. Both parents completed a questionnaire and skin testing to common inhalant allergens. Children were followed prospectively and attended review clinics at age 3 and 5 years ($\pm$4 weeks).

Children were defined as having *current wheeze* if they had wheezing in the previous 12 months at age 5. In addition, we assigned children into different wheeze phenotypes according to the history of wheeze at the two follow-ups: *no wheezing* – no wheeze during the first 3 years of life, no wheezing ever by age 5; *transient early wheeze* – wheezing during the first 3 years, no wheezing in the previous 12 months at age 5; *late-onset wheezing* – no wheeze during the first 3 years, wheezing in the previous 12 months at age 5; *persistent wheezing* – wheezing during the first 3 years, wheezing in the previous 12 months at age 5. Specific airway resistance was measured at age 5 using plethysmography. Children were asymptomatic at the time of lung function assessment.

Sensitization was ascertained by the measurement of specific IgE to mite, cat, dog, grass, milk, egg and peanut in serum collected at age 5. A mix of *S. aureus* enterotoxins consisting of SEA, SEC and TSST-1 (SE mix) was used to determine classical SE-specific IgE antibodies. Forty-nine of 510 children (9.6%) had SE mix IgE $>0.35\,kU_A/l$. Boys were more frequently SE mix sensitized than girls, and atopic children were nearly 4 times more commonly SE mix sensitive than nonatopics (19.2 vs. 5.0%, $p < 0.0001$). Analyses on the relationship between SE mix sensitization and clinically expressed allergic diseases therefore were conducted among the whole study population and atopic children only.

Whereas there was no association between rhinitis ever or current rhinitis and SE sensitization at the age of 5 years, significantly more children who had

wheezed ever were sensitized to the SE mix compared to those who have never wheezed. Similarly, current wheezers were more frequently sensitized to the SE mix, as were the children with physician-diagnosed asthma. SE mix sensitization rates appeared to increase with increasing wheeze frequency (no wheeze during last year, 1–3 attacks/year, and 4 or more attacks/year, 8.2, 11.8, and 20%, respectively, $p = 0.04$). Using never wheezers as a reference, the SE mix sensitization rate was significantly more prevalent amongst persistent wheezers, but not other types.

There was no significant difference in the baseline lung function (both specific airway resistance and FEV_1) or airway reactivity between subjects sensitized to the SE mix and those who were not sensitized. However, SE mix sensitization among current wheezers was associated with significantly higher airway reactivity.

This is the first study to suggest a relationship between SE sensitization and the presence and severity of wheeze in preschool children. Amongst preschool wheeze phenotypes, persistent wheezers were most commonly sensitized to SE, also after adjusting for the presence of eczema [59]. The association between SE sensitization and the severity of the lower airway symptoms is further emphasized by our finding on the objective measure of airway reactivity. These data suggest that SEs should be considered as disease modifiers already early in life.

Clinical Implications and Perspectives

In summary, there is accumulating evidence that superantigens, primarily derived from *S. aureus*, may have a major impact on upper and lower airway disease such as nasal polyposis and asthma, COPD and early wheezing. Superantigens at least appear to modify, if not cause, severe airway disease. SEs may furthermore affect treatment possibilities, as it was shown that these compounds may alter steroid sensitivity and expression of glucocorticoid receptor β [62]. Dexamethasone caused a 99% inhibition of phytohemagglutinin-induced PBMC proliferation, but only a 19% inhibition of the SEB-induced, 26% inhibition of the TSST-1-induced, and 29% inhibition of the SEE-induced PBMC proliferation, demonstrating that superantigens can induce steroid insensitivity. At the same time, stimulation of normal PBMCs with SEB induced a significant increase in glucocorticoid receptor β compared with phytohemagglutinin-stimulated and unstimulated cells, a possible mechanism to induce glucocorticoid insensitivity.

For diagnostic purposes, *S. aureus* can be detected in the middle nasal meatus by swabs, but would only poorly predict production of and immune

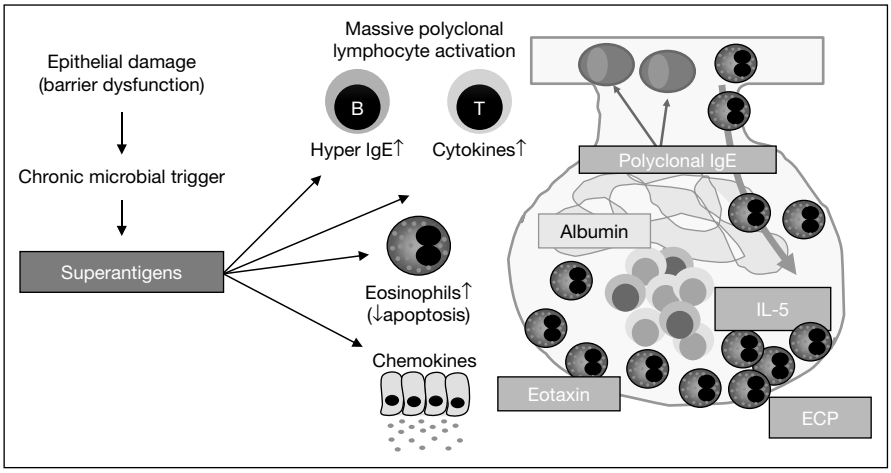

Fig. 1. The superantigen hypothesis: Superantigens, once passed the epithelial barriere, induce polyclonal T-cell and B-cell activiation and a Th2-biased eosinophilic inflammation in the airways.

response to its enterotoxins. The potential production of enterotoxins by these germs, once cultured, can be shown by PCR or protein assays, but clinical studies to show the clinical relevance in an individual patient have not yet been performed. The ability to produce enterotoxins by a given germ may also vary due to varying conditions in the nasal environment or number of colonies present. In contrast, the presence of IgE antibodies to SEs indicates a former or present stimulation of the local immune system by the respective enterotoxin, and can be tested in tissue homogenates. A polyclonal IgE response, high total IgE and increased eosinophilic mediators (ECP) would indicate the activity of the superantigens. Furthermore, Staphylococcus can now be stained intraepithelially by immuno-histochemistry; however, a positive staining would again not necessarily predict a specific immune response.

With the role of *S. aureus* as disease modifier being established, a number of therapeutic approaches may be considered (fig. 2). The potential therapeutic effect of a treatment to eradicate *S. aureus* in polyp disease or asthma has not been studied yet, but large-scale double-blind placebo-controlled randomized studies are currently ongoing. From atopic dermatitis, a disease sharing the modifying effects of staphylococcal superantigens on inflammation and disease severity, we can deduce therapeutic approaches. The skin of up to 100% of patients with atopic dermatitis is colonized with *S. aureus*, of which up to 65% have been shown to produce enterotoxins with superantigenic properties. Ten patients were treated orally with antibiotics, chlorhexidine ointment was

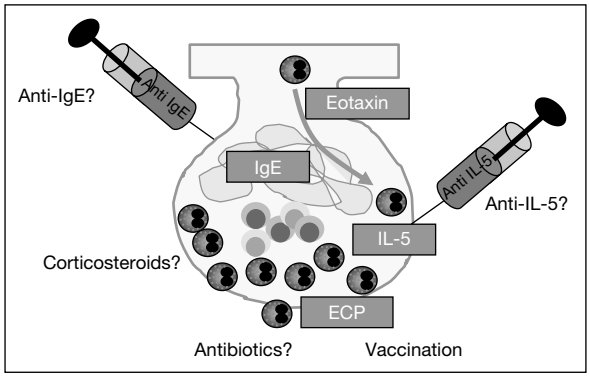

Fig. 2. Possible therapeutic approaches beyond corticosteroids.

applied to the skin, and the anterior nares were treated with mupirocin ointment, and a bath containing potassium permanganate was taken daily [63]. In addition, their partners were treated topically. The severity scoring of atopic dermatitis decreased in 9 of 10 patients who received antimicrobial treatment, and this effect was more pronounced in patients with a higher baseline severity scoring of atopic dermatitis. Thus, antimicrobial treatment led to a significant, albeit temporary improvement of atopic dermatitis in patients who were colonized with *S. aureus.* A similar effect may be anticipated for NPs, however, this needs to be confirmed. Other approaches, such as long-term antibiotic treatment with intracellular activity, in combination with corticosteroids to decrease the immune response and increase steroid sensitivity, might be developed in the future for sustained treatment success.

Considering the marked local production of IgE antibodies in NPs and its relation to the severity of disease, strategies to antagonize IgE antibodies could be of relevance. Double-blind placebo-controlled randomized studies with anti-IgE treatment are not yet available in CRS with and without NPs. In view of the prominence of eosinophilic inflammation and increased IL-5 levels associated with colonization with *S. aureus* in NPs, it is likely that a better understanding of the mechanisms underlying the migration, activation and maintenance of eosinophils in NP tissue will be key to understanding the etiology and pathogenesis of NPs. In this context, treatment strategies antagonizing IL-5 offers the ultimate opportunity to test the IL-5/eosinophil hypothesis in nasal polyposis. Recently, Gevaert et al. [64] have demonstrated shrinkage of NPs in half of the treated patients for up to 4 weeks after intravenous injection of a single dose of an anti-human IL-5 monoclonal antibody. These insights into the regulation of IL-5 and eosinophilia in NPs (re-)open therapeutic perspectives in nasal

polyposis based on eosinophil-selective targets and emphasize the role of eosinophils in nasal polyposis.

Finally, vaccination therapy against *S. aureus* would be the ultimate step in order to reduce colonization and treat superantigen-modified diseases.

Acknowledgements

This work was supported by grants to C.B. from the Flemish Scientific Research Board, FWO No. A12/5-HB-KH3 and FWO No. F6/15-DP.D7675, and to P.G., FWO No. A2/5-LG-A4648.

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Author Index

Subject Index